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Prodrugs of weak acids with activity against *M.tuberculosis*

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Resumo

A tuberculose é uma doença infecciosa que afecta milhoões de pessoas em todo o mundo. Em 2015 foram relatados 9,6 milhões de novos casos. O agente infeccioso causador da doença, *Mycobacterium tuberculosis*, desenvolveu várias resistências aos tratamentos existentes. Esta bactéria possui um envelope celular bastante complexo, constituído por ácidos micólicos interferindo na sensibilidade da bactéria aos antiobióticos hidrofóbicos. Os tratamentos actualmente existentes consistem em combinações de fármacos de primeira linha (isoniazida, rifampicina, pirazinamida e ethambutol). No entanto, surgiram estirpes resistentes a alguns dos fármacos administrados no tratamento dando origem a outros dois tipos da doença: tuberculose multi-resistente e tuberculose extensivamente multi-resistente. Nestes casos o tratamento envolve outro tipo de fármacos: fármacos de 2ª e 3ª linha. Estes tipos de fármacos são mais dispendiosos e apresentam diversos efeitos secundários.

Nos fármacos de 2ª linha, existe um grupo de fármacos que são as fluroquinolonas. Estes fármacos derivam do ácido nalidíxico e contêm fluor na sua estrutura, sendo activos contra um grande número de bactérias. Este grupo de compostos tem como alvo enzimas envolvidos no processo de transcrição do DNA, processo crucial para a vitalidade da bactéria. O ácido nalidíxico é uma quinolona usada normalmente no tratamento de infeções urinárias causadas por bactérias. Este antibiótico actua através da inibição do ADN girase, um enzima fulcral no processo de transcrição do ADN bacteriano.

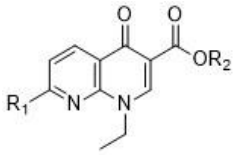
Estudos anteriores demonstraram que alguns ácidos fracos apresentam atividade antimicobacteriana contra *Mycobacterium tuberculosis* em virtude da sua acumulação no interior da célula, devido a um mecanismo de efluxo deficiente. Sendo o ácido nalidíxico um ácido fraco, alterações na sua estrutura poderão melhorar as suas propriedades farmacocinéticas bem como a sua actividade antimicobacteriana.

Os pró-fármacos são compostos que não são biologicamente activos, ou seja, necessitam de ser activados através de reações químicas ou enzimáticas. Este tipo de compostos é sintetizado quando a substância activa possui propriedades indesejadas, como baixa absorção, degradação rápida por parte de enzimas, deficiente entrada nas células das micobactérias, entre outras.

Na preparação de um fármaco contra a tuberculose, é necessário que este apresente boas propriedades de absorção, distribuição, metabolismo e excrecção, sendo ativo no local desejado e não tóxico contra o organismo humano. Neste sentido, é necessária a síntese de compostos estáveis à hidrólise por parte de enzimas, plasmáticos e hepáticos do hospedeiro, que penetrem a membrana celular da bacteria, portanto lipofílicos e que sejam activos contra a bactéria *Mycobacterium tuberculosis*. Para serem ativos, é necessário que os compostos sejam hidrolisados pelos enzimas da micobácteria, de forma a converter os pró-fármacos na sua forma activa. Por último, os derivados sintetizados devem apresentar níveis de toxicidade reduzidos para o hospedeiro.

Uma das estratégias mais comuns na síntese de pró-fármacos é a formação de grupos éster pois estes grupos levam a um aumento da lipofilia do composto e consequentemente facilitam a sua passagem através das membranas biológicas. Estudos anteriores demonstraram que o *Mycobacterium tuberculosis* possui enzimas responsáveis pela hidrólise de ésteres.

Neste trabalho foram sintetizados derivados do ácido nalidíxico (1). Os derivados sintetizados são ésteres pois estes são mais lipofílicos, e por isso penetrarão mais facilmente nas micobactérias que o ácido correspondente. Para que os compostos sejam ativos, é necessário que cheguem ao local de acção sem sofrer alterações na sua estrutura. Assim, é necessário que estes ésteres sejam resistentes à hidrólise por enzimas humanos e, por outro lado, susceptíveis à hidrólise por parte de enzimas micobacterianos.



R_1: CH_3, CCl_3
R_2: different alkoxyl chains

1

Os ésteres sintetizados apresentam diferentes cadeias alcoxílicas (entre 2 e 14 carbonos), de forma a estudar o efeito da lipofilia na estabilidade e actividade destes. Na porção acilo, foi feita uma substituição em relação ao ácido nalidíxico de forma a verificar se aquela porção da molécula tem influência na actividade do composto. Pretendeu-se avaliar se a substituição do grupo C_7-CH_3 pelo grupo C_7-CCl_3 levava a melhores resultados a nível de inibição do crescimento micobacteriano bem como se os compostos eram susceptíveis ou resistentes à hidrólise enzimática. De facto, estudos anteriores demonstram que aquela porção da molécula apresenta um papel crucial na actividade deste tipo de compostos. Para além de ésteres, foi sintetizado também um derivado do ácido carboxílico que continha apenas a substituição do grupo C_7-CH_3 pelo grupo C_7-CCl_3, de forma a testar a sua actividade bem como para controlo de ensaios de estabilidade.

Na síntese de todos os derivados, dois métodos distintos foram usados e observou-se que os rendimentos eram bastante diferentes entre os dois grupos de ésteres: ésteres com o grupo C_7-CH_3 tiveram um rendimento de síntese acima dos 51% enquanto que ésteres com o grupo C_7-CCl_3 tiveram um rendimento de síntese inferior a 35%.

Os estudos de actividade foram realizados na estirpe H_{37}Rv de *Mycobacterium tuberculosis*. Nestes estudos, foram analisadas as concentrações mínimas necessárias para inibição de 90% e de pelo menos 50% do crescimento bacteriano. Também foi analisada a percentagem de inibição do crescimento bacteriano para uma concentração administrada de composto de 100 µM. Observou-se que praticamente todos os derivados do ácido nalidíxico inibem o crescimento micobacteriano. Para além disso, verificou-se que os derivados com o substituinte C_7–CCl_3 são mais activos, de um modo geral, que os derivados com o substituinte C_7-CH_3.

Foram realizados estudos de estabilidade em plasma humano e em tampão fosfato pH 7,4. Os estudos em plasma humano permitiram observar se os compostos são susceptíveis à hidrólise enzimática por partes dos enzimas presentes neste compartimento corporal. Os ensaios em tampão fosfato pH 7,4 foram realizados de forma a avaliar a estabilidade química dos compostos. Verificou-se que os derivados sintetizados são muito resistentes à hidrólise química, bem como à hidrólise enzimática. De facto, os compostos apresentam tempos de semi-vida bastante elevados revelando-se muito estáveis. Os resultados obtidos demonstram que os compostos sintetizados apresentam tempos de semi-vida superiores a 2,8 dias, no caso da hidrólise enzimática, e 7,8 dias no caso da hidrólise química. Para além disso, no estudo da susceptibilidade à hidrólise

enzimática, houve compostos que não demonstraram degradação durante as 72 horas do ensaio em plasma humano.

Fez-se também uma comparação entre as constantes cinéticas da hidrólise enzimática e da hidrólise química para avaliar se a hidrólise química tem impacto nos estudos de estabilidade em plasma humano. Verificou-se que para alguns compostos este tipo de hidrólise não é significativa, porém, em alguns deles, esta apresenta um impacto semelhante ao da hidrólise enzimática. Com base neste estudo, pretende-se o desenvolvimento de novos pró-fármacos com actividade contra a tuberculose, de modo a combater as estirpes resistentes aos tratamentos atuais.

Neste trabalho, foi possível concluir que os derivados ésteres desenvolvidos do ácido nalidíxico são muito estáveis à hidrólise enzimática bem como à hidrólise química. Verificou-se que os derivados que apresentam o substituinte C_7–CCl_3 são mais activos contra a estirpe estudada do que os compostos que apresentam o substituinte C_7-CH_3. Apesar de praticamente todos os derivados sintetizados apresentaram actividade contra a bacteria, alterações na sua estrutura são necessárias de modo a aumentar a inibição do crescimento bacteriano, a menores concentrações administradas, mas mantendo a estabilidade à hidrólise enzimática no hospedeiro.

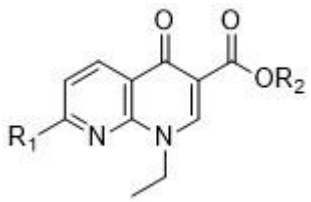
No futuro mais estudos com este tipo de derivados serão necessários, tais como, ensaios de toxicidade, estudos de modelação, estudos em homogenato de fígado, entre outros. Para além disso, é necessário compreender qual o mecanismo que leva à inibição do crescimento bacteriano por parte dos derivados do ácido nalidíxico sintetizados.

Palavras-chave: Tuberculose, Ácido nalidíxico, Pró-fármacos, Estabilidade, Actividade

Abstract

Tuberculosis is one of the most prevelent diseases in the world with high rates of incindence and mortality. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, is one of the leading bacterial cause of infection. There are several treatment regiments available to treat *Mycobacterium tuberculosis* but the development of new strains and co-infection with HIV led to the need of new antibiotics to treat this pathogen. One of the antibiotics to treat multidrug resistant and extensively drug resistant tuberculosis are fluoroquinolones. These drugs are fluorinated nalidixic acid derivatives and are active against a broad spectrum of bacteria.

Nalidixic acid is a quinolone used in the treatment of urinary tract infections caused by bacteria. This drug inhibitis DNA gyrase, an enzyme with an important role in bacterial transcription. Many organic acid drugs have small half lives, high protein binding and difficulties in celular penetration. In order to test if we could obtain derivatives with better activity/improved pharmacokinetics several ester derivatives (1) were synthesized.These derivatives will be converted inside the bacterial cell to their correspondent acids. In order to be effective, these compounds need to be resistant to hydrolysis by human enzymes, but must be hydrolyzed by the mycobacterial enzymes.



R_1: CH_3, CCl_3
R_2: different alkoxyl chains

1

Almost all of the synthesized derivatives have shown antibacterial activity against *M. tuberculosis* H_{37}Rv strain. Besides that, it was shown that derivatives containing the C_7-CCl_3 group instead of a C_7-CH_3 group inhibit more the bacterial growth.

Stability assays were performed in human plasma and phosphate buffer pH 7.4 in order to evaluate enzymatic and chemical hydrolysis, respectively. With these assays, it was possible to observe that all the studied compounds have a good stability, being resistant to the action of the enzymes in human plasma. Besides that, it is possible to conclude that the synthesized compounds are also very resistant to chemical hydrolysis in phosphate buffer pH 7.4.

In the present work it was possible to conclude that nalidixic acid derivatives are very stable to enzymatic and chemical hydrolysis. It was also observed that almost all the synthesized compounds are active against *Mycobacterium tuberculosis* and changes in the acyl structure lead to a higher inhibition of bacterial growth. Although, further changes in the structure can probably improve further the activity of the compounds while keeping the high stability to host enzymatic hydrolysis.

Keywords: Tuberculosis, Nalidixic acid, Prodrugs, Stability, Activity

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List of Abbreviations

TB – Tuberculosis

***M. tuberculosis* –** *Mycobacterium tuberculosis*

NTM - Non-tuberculous mycobacteria

MTC – *Mycobacterium tuberculosis* complex

MDR-TB – Multidrug resistance tuberculosis

XDR-TB – Extensively drug-resistance tuberculosis

FQ – Fluoroquinolones

CIP – Ciprofloxacin

LEV – Levofloxacin

MOX – Moxifloxacin

NAL –Nalidixic acid

QRDR - Quinolone resistance-determining region

ADMET – Absorption, distribution, metabolism, excretation and toxicity

Ser – Serine

His – Histidine

Gly – Glycine

Leu – Leucine

BChE – Butyrylcholinesterase

PON 1 – Paraoxonase 1

PON 2 – Paraoxonase 2

PON 3 – Paraoxonase 3

HAS – Human serum albumin

Tyr – Tyrosine

LipF - Rv3487c

LipH – Rv1399

Eq – Equivalent

h – hour

^{1}H-NMR – Proton nuclear magnetic ressonance

^{13}C-NMR – Carbon nuclear magnetic ressonance

NMR – Nuclear magnetic ressonance

HMQC – Heteronuclear multiple-quantum correlation

HMBC – Heteronuclear multiple-bond correlation

APT – Attached proton test

***J* –** Coupling constant

HPLC – High performance liquid chromatography

ACN – Acetonitrile

k_{obs} **–** pseudo-first order constant

$t_{1/2}$ **–** half time live

MIC – Minimum inhibitiory concentration

I – inductive effect

Asn – Asparagine

Lys – Lysine

logP – Partition coefficient

Introduction

1. Introduction

1.1 Tuberculosis

1.1.1 Epidemology

Tuberculosis (TB) is a major global health problem that infects nearly one-third of the worlds population[1]. In 2014, there were an estimated 9.6 million new TB cases and 1.5 million TB deaths (1.1 million among HIV-negative persons and 0.4 million among HIV-positive persons)[2]. In **figure 1** is shown the estimated TB incidence rates in 2014 and since this number and the number of TB deaths are unacceptable there is an emergence to find new alternatives for treatment[2].

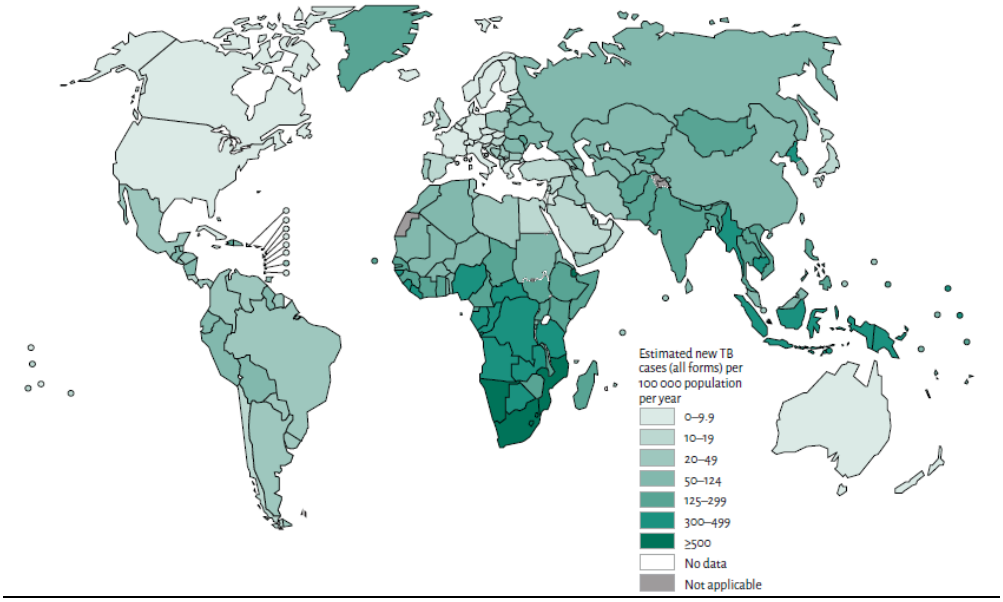


Figure 1. 1 - Estimated TB incidence rates in 2014[2].

1.1.2 Disease Characterization

TB in an infectious disease caused by *Mycobacterium tuberculosis* (*M. tuberculosis*). The infection starts usually by the inhalation of droplet nuclei (1-5 μm in diameter particles) containing *M. tuberculosis*, expectorated when patients with active TB cough. The inhaled droplet nuclei avoid defenses of the bronchi due to their small size and penetrate into terminal alveoli of the lungs where they are engulfed by non-specifically activated alveolar macrophages, lung macrophages and dendritic cells. If the macrophages are unable to destroy mycobacteria, they continue to multiply until the macrophages rupture. The released organisms are subsequently ingested by inactivated blood macrophages that are attracted to the lung by chemotactic factors[3,4]. In the lungs, *M. tuberculosis* can also infect endothelial cells, M cells and type 1 and 2 epithelial cells, which means that *M. tuberculosis* can infect non-phagocytic cells also[3].

In the initial phase of the infection, *M. tuberculosis* internalized by macrophages and dendritic cells replicates intracellularly and immune cells, infected with mycobacteria, can cross the alveolar barrier and cause systemic dissemination[5].

The typical pattern of TB occurs in four stages. The first stage occurs in 3-8 weeks and corresponds to the implementation of inhaled mycobacteria in alveoli. This is followed by the dissemination through the lymphatic circulation to the regional lymph nodes in the lungs which results in the formation of the primary complex. In the second stage, the mycobacteria circulate through the bloodstream to other organs. This stage occurs in the ensuing approximate 3 months and some patients suffer fatal disease during this time. The third stage can occur at any time up to two years of infection and is marked by severe chest pain and inflammation of the pleural surface. The final stage consists in the resolution of the primary complex and can take several years and, in some cases, extrapulmonary disease can become manifest during this time[6].

1.1.3 *Mycobacterium tuberculosis*

Mycobacterium is a bacteria genus responsible for several diseases, such as TB and leprae. They are acid-fast bacilli, aerobic obligate, immobile and with a cell wall rich in lipid content[7].

The scientific classification is:

Kingdom: Bacteria
Phylum: Actinobacteria
Class: Actinobacteria
Order: Corynebacterineae
Family: Mycobacteriaceae
Genus: *Mycobacterium*[8]

These bacteria are divided in 2 groups: Non-tuberculous mycobacteria (NTM) and *Mycobacterium tuberculosis* complex (MTC). The NTM species are ubiquitous in nature, they are considered opportunistic pathogens and several species are associated with human diseases[9]. Species like *M. avium*, *M. smegmatis* and *M. ulcerans* belong to this group[10,11]. In contrast, MTC is always considered pathogen and this group includes: *M. tuberculosis*, *M. bovis* and *M. caprae*[10,12].

The most pathogenic bacteria of this genus are *M. tuberculosis*, that causes tuberculosis, *M. leprae* that is the causative agent of leprosy and *M. ulcerans*, that causes ulcer, a serious superficial infection[4]. *M. tuberculosis* grows very slowly and has a complex cell envelop that is constituted with mycolic acids[13,14]. Mycolic acids are very long-chain branched fatty acids attached to the cell wall. They are an important determinant of fluidity of the cell wall and they are also related to the sensitivity of the mycobacteria species to hydrophobic antibiotics[13,15]. The generation time of *M. tuberculosis* in infected animals is typically 24h. The state of dormancy in which the bacillus remains inactive within infected tissue results from the action of the cell-mediated immune response that can't eradicate the infection[16]. In order to prevent the growth of *M. tuberculosis* during persistent infections, the strategy used by the host consists in forming granulomas (aggregates of immune cells around infected tissues)[17]. The mycobacteria can stay in this state of dormancy for decades and later a failure of the immune system may permit their revival and the activation of the disease[18].

There is no simple answer to what makes *M. tuberculosis* virulent because this bacteria does not have classical virulence factors like other bacterial pathogens e.g. produced toxins, so it is still necessary to find factors that are important for the progression of TB and therefore to a better understanding of this disease[19,20]. There is still limited knowledge of how *M. tuberculosis* causes disease but so far the standard terms "mortality" and "morbidity" have been used for a description of *M. tuberculosis* virulence and then it is important to understand the pathogenesis associated with TB[20].

1.1.4 Treatment

The current recommended treatment of TB consists in a drug combination. Treatment requires a minimum of 6 months duration in 2 phases: 2 months of 4 first-line drugs (isoniazid, rifampicin, pyrazinamide and ethambutol) in the intensive phase followed by 4 months of isoniazid plus rifampicin in the continuous stage[21,22].

Improper prescriptions and patient noncompliance has led to the emergence of new strains of *M. tuberculosis* resistant to some or all current antitubercular drugs[23]. Multidrug resistance tuberculosis (MDR-TB) is defined as tuberculosis resistant to isoniazid and rifampicin and is associated with high death rates of 50% to 80%[23,24]. Treatment regiments of MDR-TB include at least 4 second-line drugs and total duration therapy can go up to 28 months[21]. Extensively drug-resistance tuberculosis (XDR-TB) is another type of resistant TB, that is resistant to isoniazid, rifampicin, one fluroquinolone and to at least one of the three injectable second-line drugs that is associated with high mortality rates, takes substantially longer to treat than MDR-TB and requires the use of third-line anti-TB drugs, which are expensive and often with more side effects than first-line and second-line drugs[21].

In **table 1.1** is presented some of the drugs used nowadays in the treatment of TB.

Table 1. 1 - Drugs used in TB treatment[21,25–28].

Drug	Class	Administration	Effect
INH	1st line	Oral	Inhibits mycolic acid synthesis
RIF	1st line	Oral	Inhibits transcription
PZA	1st line	Oral	Acidifies cytoplasm
EMB	1st line	Oral	Inhibits lipids biosynthesis
STM	2nd line	Injectable	Inhibits protein synthesis
CAP	2nd line	Injectable	Inhibits protein synthesis
AMI	2nd line	Injectable	Inhibits protein synthesis
KAN	2nd line	Injectable	Inhibits protein synthesis

Drug	Class	Administration	Effect
OFX	2nd line	Oral	Inhibits DNA supercoiling
CYC	2nd line	Oral	Inhibits peptidoglycan synthesis
CFZ	3rd line	Oral	Interact with bacterial DNA
AMX/CLV	3rd line	Oral	Inhibition of β-lactamase
IPM/CLN	3rd line	Injectable	IPM – Inhibition of β-lactamase CLN – Inhibition of enzyme responsible for degradation IPM

INH – isoniazid; **RIF** – rifampicin; **PZA** – pyrazinamide; **EMB** – ethambutol; **STM** – streptomycin; **CAP** – capreomycin; **AMI** – amikacin; **KAN** – kanamycin; **OFX** – ofloxacin; **CYC** – cycloserine; **CFZ** – clofazimine; **AMX/CLV** – amoxicillin plus clavulanate; **IPM/CLN** – imipenem plus cilastatin.

1.1.5 Fluoroquinolones

Fluoroquinolones (FQ), fluorine-containing nalidixic acid derivatives (**Figure 1.2**), are a group of compounds that target DNA gyrase and DNA topoisomerase II and have a broad-spectrum of antimicrobial activity[21,29]. These compounds are recommended and widely used in the treatment of bacterial infections of the respiratory, gastrointestinal, and urinary tracts, as well as sexually transmitted diseases and chronic osteomyelitis. It has been shown that FQ can also penetrate into macrophages and have bactericidal activity there[29]. Besides that, FQ have shown potent activity against *M. tuberculosis* and are used as second-line drugs in TB therapy[30,31].

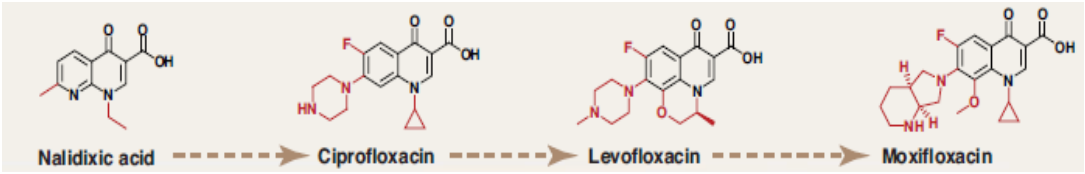


Figure 1. 2 - Four generations of quinolones. Ciprofloxacin, levofloxacin and moxifloxacin are FQ. Scaffolds are colored black and peripheral chemical modifications are colored red[32].

Ciprofloxacin (CIP), an inhibitor of DNA gyrase, is used in the most potent fluoroquinolone and is active against a broad range of bacteria and the most susceptible are aerobic gram-negative bacilli[33,34].CIP was the first fluoroquinolone indicated for infections outside the urinary tract and was considered a major advance in therapy for aerobic gram-negative infections. However, CIP was noted to have poor activity against anaerobes[35]. This drug can also interact with a number of drugs, some herbal and natural supplements, and certain thyroid medications[34].

Levofloxacin (LEV) is an isomer of ofloxacin (used in treatment of MDR-TB) and has increased *in vitro* antibacterial activity against a variety of bacteria including anaerobes[36]. LEV is also used in the treatment of MDR-TB if the strain is susceptible or if the agent is thought to have efficacy[31]. The mode of action of this drug is the inhibition of DNA gyrase and DNA topoisomerase II[37].

Moxifloxacin (MOX) is synthetic fluoroquinolone antibiotic agent used in the treatment of sinus and lung infections. The bactericidal action of these drugs results from inhibition of DNA gyrase and DNA topoisomerase II[38]. MOX is also a second-line drug and is the most potent fluoroquinolone used in TB treatment although is less active than isoniazid (first-line drug)[31,39].

1.1.6 Nalidixic Acid

Nalidixic acid (NAL) (**figure 1.3**) was the first member of the quinolone family of antibacterial agents synthesized and it's used in the treatment of urinary tract infections caused my susceptible gram-negative microorganisms[40,41]. NAL is administrated orally, is rapidly absorbed from the gastrointestinal tract and has a bioavailability of approximately 96%. NAL metabolism is hepatic and 30% of the administrated dose is converted to the active metabolite, hydroxynalidixic acid (**Figure 1.4**)[42].

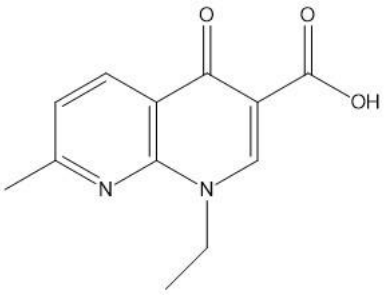


Figure 1. 3 - NAL structure.

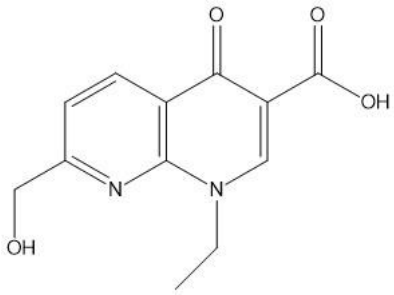


Figure 1. 4 - Hydroxynalidixic acid structure.

NAL is an antibiotic that inhibits DNA gyrase, an enzyme that catalyses the negative supercoiling of DNA, that is essential for DNA replication, transcription and recombination[43,44]. This enzyme is constituted by two A subunits and two B subunits, encoded by *gryA* and *gryB*, respectively[44].Quinolone sensitivity is controlled by subunits A, which contain the breakage-reunion active site while subunits B promote ATP hydrolysis, needed for energy transduction[23,44].

It was shown that fluoroquinolones had the potential to reduce the duration of therapy in TB murine models[21]. NAL and other quinolones were previously tested in *M. tuberculosis* with a mutation in *gryA* and have shown poor DNA cleavage stimulation[44]. These mutations occur in a conserved region that is called quinolone resistance-determining region (QRDR) of the *gyrA* and *gyrB* genes. Mutations within the QRDR are associated with quinolones resistance[29].

1.2 Prodrugs

Prodrugs are compounds that are not biologically active and have to be transformed into active products by enzymatic or chemical reactions[45]. The active compounds have undesirable properties that may become pharmacological, pharmaceutical or pharmacokinetic barriers in clinical drug application. Using drug derivatization with retention of the desirable therapeutic activity has an important role on improving drug efficacy[46]. Optimizing drug activity is a two-dimensional problem because the activity at the target is essential but is just as important as the efficient delivery of the agent in that same target[47].

Prodrugs can be applied to achieve increased chemical or metabolic stability, higher water solubility or higher solubility in lipid membranes, reduced toxicity, improved oral or local absorption[45]. The main objectives of prodrugs are:

- Modification of drug pharmacokinetics;
- Longer action;
- Decrease in toxicity and side-effects;
- Increase in selectivity;
- Resolution of formulation problems, such as stability, solubility and organoleptic properties[48].

Prodrugs can be classified in two main classes: carrier-linked prodrugs and bioprecursor prodrugs. In carrier-linked prodrugs, the active compound is temporary linked to a carrier through a bioreversible covalent linkage. Once in the body, the carrier-linked prodrug undergoes biotransformation, releasing the drug and the carrier. The carrier should be nonimmunogenic, easy to synthesize at a low cost, stable under the conditions of prodrug administration and undergo biodegradation to nonactive metabolites. This class includes a group of prodrugs called co-drugs that are prodrugs with two pharmacologically active agents coupled together into a single molecule. Bioprecursors are prodrugs that result from a molecular modification of the active compound itself. The bioprecursor prodrug is transformed metabolically or chemically by hydration, oxidation or reduction into the active agent[49].

Prodrugs can also be classified in two another types, depending on the site of conversion to the active metabolite: Type I prodrugs are metabolized intracellularly (Type IA, at the cellular targets of their therapeutic action and Type IB, by metabolic tissues), whereas Type II prodrugs are metabolized extracellularly (Type IIA, in the milieu of the gastrointestinal fluid; Type IIB, in the circulatory system and/or other extracellular fluid compartments and Type IIC, near or inside therapeutic target/cells)[49,50].

Some of the first- and second-line agents used in the treatment of TB are prodrugs (for example, isoniazid and pyrazinamide). These antimycobacterial prodrugs can be divided in two main groups depending on their mechanism of action: mycobacteria bioactivated and host bioactivated. The mycobacteria bioactivated prodrugs have increased bioavailability and solubility, and decreased toxicity. The main benefits of host-bioactivated drugs are: increased bioavailability and therapeutic effectiveness; improved solubility, chemical stability; increased organ/tissue-selective delivery of the active agent and decreased toxicity. However, the main problem of antimycobacterial prodrugs, already used in TB treatment, is the emergence of strains resistant to prodrugs due to mutations in the gene encoding the activator[50].

1.2.1 Esters as Prodrugs

The prodrug strategy is one of the most promising approaches to enhance the therapeutic efficacy of pharmacologically active agents by optimizing the absorption, distribution, metabolism, excretion and toxicity (ADMET) properties of the parent drugs. This is a faster strategy than searching for entirely new therapeutic agents with suitable ADMET characteristics[50].

Prodrug design is used to improve the active drugs and some of the most common functional groups, such as carboxylic, hydroxyl, amine, phosphate and carbonyl are transformed in esters, carbonates, carbamates, amides and phosphates.[51]

The features of an ideal prodrug include: hydrolysis resistance during absorption; weak or no activity; aqueous solubility; good permeability through cells; chemical stability at different pH and kinetics that allow the release of the parental drug. Among the chemical bonds used to link the parental drug and carrier, esters have proven to be promising due to their amenability to hydrolysis both *in vivo* and *in vitro*[52].

Esters of active agents with carboxyl, hydroxyl or thiol functionalities are the most commonly used prodrugs because these prodrugs enhance the lipophilicity, thus the passive membrane permeability, and decrease the water solubility by masking charged groups[49,51]. An ideal ester prodrug should have chemical stability across pH range, high aqueous solubility and exhibit good transcellular absorption phase[49].

1.2.2 Chemical Hydrolysis

Carboxylic acid derivatives hydrolysis can be catalysed by acids or bases. The hydrolysis mechanisms, in both acid- and base-promoted, involve a nucleophilic addition-elimination at the acyl carbon[53].

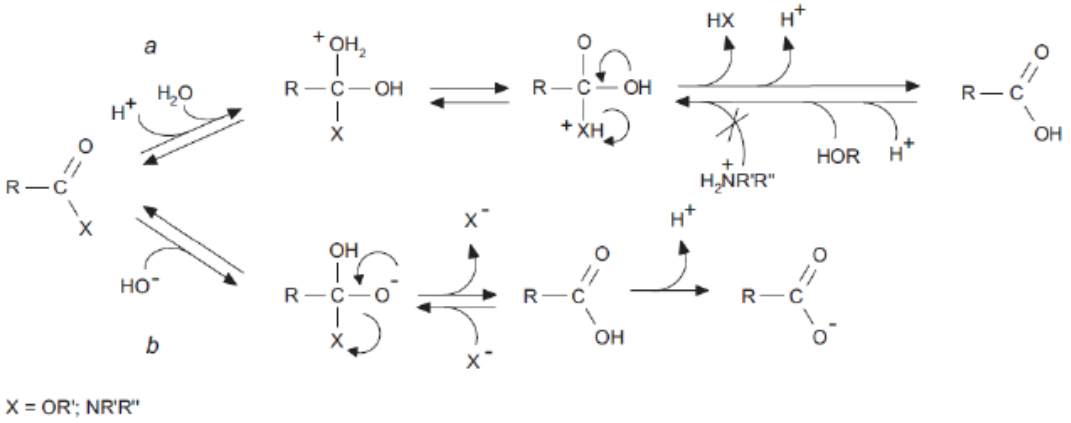


Figure 1. 3 - Scheme of chemical hydrolysis of carboxylic derivatives (esters and amides). (a) acid catalysis (b) base catalysis[54].

In acid catalysis (**Figure 1.5a**), the first step is the protonation of the ester carbonyl to make it more electrophilic. Then, the water oxygen functions as the nucleophile attacking the electrophilic carbon in the **C=O**, creating the tetrahedral intermediate. The oxygen from the water is deprotonated to neutralise the charge and the **XH** group is protonated to convert it into a good leaving group: the carboxylic acid product is obtained and the acid catalyst is regenerated[53–55].

In base hydrolysis (**Figure 1.5b**), the hydroxy nucleophile attacks at the electrophilic carbon of the ester **C=O**, creating the tetrahedral intermediate. Then, the intermediate expels an alkoxyl ion, leading to the carboxylic acid. After, transfer of a proton leads to the products of the reaction[53,55].

1.2.3 Enzymatic Hydrolysis

The enzymatic hydrolysis is a common hydrolysis reaction that is catalyzed by an enzyme. Enzymatic hydrolysis is more efficient than chemical hydrolysis due to the decrease in the Gibbs energy of the transition state[54]. Esters and amides, endogenous or exogenous, are one of the major substrates for

hydrolases, enzymes of class 3 that catalyze the attack to functional groups by water molecules. Hydrolases have three catalytic features at the active site that accelerate the rate of hydrolysis (**Figure 1.6**)[54,56]. These catalytic sites are: an electrophilic component, which increases the polarization of the carbonyl group in the substrate (**Z**$^{+}$); a nucleophile to attack the carbonyl C-atom, leading to the formation of a tetrahedral intermediate (**Y:**) and a proton donor to transform -OR' and –NR'R'' into better leaving groups (**H-B**)[54].

Figure 1. 4 - Catalytic groups of hydrolases involved in ester and amide bond hydrolysis[54].

Based on the structures of their catalytic sites, hydrolases can be divided in five classes: serine hydrolases, threonine hydrolases, cysteine hydrolases, aspartic hydrolases and metallohydrolases[54]. Hydrolases have different substrates such as: peptides, ethers, esters, glycosylases, acid anhydrides and halide bonds[57].

1.3 Esterases

Esterases are enzymes that catalyze the cleavage and formation of ester bonds. These enzymes belong to hydrolases class of enzyme, EC 3 and can act as hydrolyses when catalyzing the cleavage of ester bonds[57,58]. Within the ester formation bonds esterases can catalyze three types of reactions: esterifications, interesterification and transesterification reactions with good chemo-, region- and/or enantioselectivity[58]. Esterases are subdivided in the type of reaction they catalyze:

- Carboxylic ester hydrolases (EC 3.1.1);
- Thioester hydrolases (EC 3.1.2);
- Phosphatases (EC 3.1.3);
- Phosphodiester hydrolases (EC 3.1.4);
- Triphosporic monoester hydrolases (EC 3.1.5);
- Sulfatases (EC 3.1.6);
- Diphosphoric monoesterases (EC 3.1.7);
- Phosphoric triester hydrolases (EC 3.1.8);
- Exonucleases (EC 3.1.11-16);
- Endonucleases (EC 3.1.21-31)[57].

The major carboxylic ester hydrolases classes are carboxylesterases (EC 3.1.1.1, carboxylic-ester hydrolase) and carboxylesterases (EC 3.1.1.3, triacylglycerol hydrolases)[59]. Carboxylesterases catalyze the hydrolysis of a wide variety of substrates including esters, amides and carbamates (endogenous or exogenous)[60]. These enzymes catalyze hydrolysis of esters, by the water, resulting in alcohol and carboxylate[61]. The three-dimensional structure of carboxylesterases show the characteristic α/β-

hydrolase fold[59] (**Figure 1.7**). In this structure, each parallel β-segment is attached to its neighbour by an α-helical segment[56]. The catalytic triad is composed of serine-aspartic acid-histidine (Ser-Asp-His) (**Figure 1.7**) and usually a *consensus* sequence glycine-x-x-leucine (Gly-x-x-Leu motif, where x can be any amino acid residue) is around the active site. The mechanism for ester hydrolysis is based in of four steps: first, the substrate is bound to the active serine, yielding a tetrahedral intermediate stabilized by the catalytic His and Asp residue. Then, the alcohol is released and an acyl-enzyme complex is formed. Next, attack of water forms again a new tetrahedral intermediate, which after resolution yields the acid and free enzyme[59].

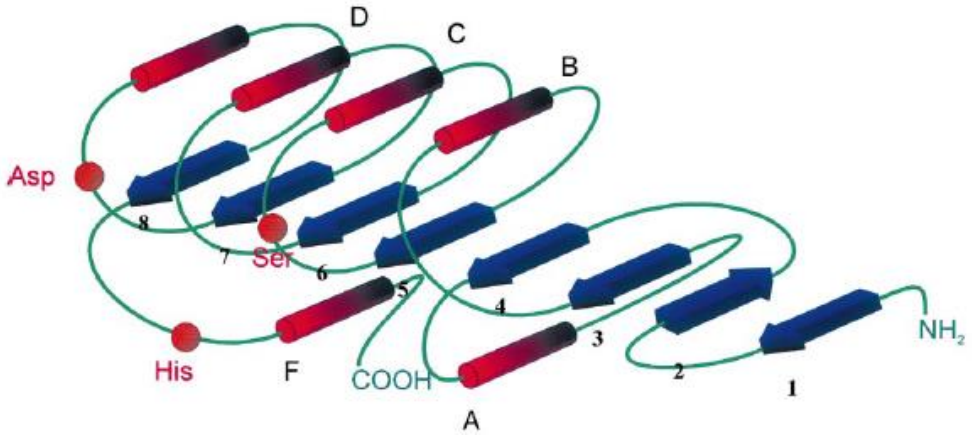


Figure 1. 5 – Scheme of the α/β-hydrolase fold. β-Sheets (1-8) are shown as blue arrows, α-helices (A-F) as red columns and the relative positions of the amino acids residues of the catalytic triad are indicated as orange circles[59].

Carboxylesterases are widely present throughout the body (e.g., intestine, brain, skin, etc.) with their highest activity being in the liver. These esterases act also as an effective biological barrier to limit the distribution of substrates that might be toxic and induce their elimination by turning them into polar molecules. Ester prodrugs are intentionally designed to be activated by these esterases since most compounds will be detoxified by them[62].

1.3.1 Esterases of Human Plasma

Human plasma esterases have an important role in drug metabolism. These enzymes participate in activation of ester prodrugs, inactivation of drugs and detoxify natural and synthetic ester-containing poisons[63].

The human plasma has three esterases groups:

- Butyrylcholinesterase (EC 3.1.1.8);
- Paraoxonase (EC 3.1.8.1);
- Acetylcholinesterase (EC 3.1.1.7)[63].

Besides this three groups, there is a very important and abundant protein in the human plasma with ester hydrolytic capacity: albumin[63].

Paraoxonases are called "A-esterases" whereas butyrylcholinesterase and acetylcholinesterase are called "B-esterases"[64].

Butyrylcholinesterase (BChE) is also known as pseudocholinesterase, non-specific cholinesterase or simply cholinesterase. This enzyme acts on butyrylcholine and acetylcholine. BChE has higher activity in liver, intestine, lungs, heart and kidney. Serum BChE is synthesized in liver and secreted into plasma, where it has higher activity[65,66]. BChE has also an important role as detoxification enzyme because it can hydrolyse compounds with carboxylic or phosphoric acid ester groups. Therefore, this enzyme can hydrolyse compounds like: succinyldicholine (neuromuscular blocking drug); cocaine; aspirin; heroin and others[65].

Paraoxonase is an enzyme family with three members: paraoxonase 1(PON1), paraoxonase 2 (PON2) and paraoxonase 3 (PON3). These esterases are capable of hydrolysing organophosphates as well as aromatic esters[64]. PON1 and PON3 can be found in the cholesterol-carrying particles HDL and can inhibit lipid oxidation in LDL whereas PON2 is found in many tissues[67]. Only PON1 is present in human plasma. PON1 is a proefficient esterase toward several synthetic substrates whereas PON2 and PON3 exhibit high lactonase activity[64].

Acetylcholinesterase (AChE) or "true cholinesterase" is known to be abundant in brain, muscle and erythrocyte membrane being present in negligible amounts in human plasma[63,65]. Like BChE, AChE belongs to the α/β hydrolase-fold family[66]. The main function of AChE is hydrolysis of the neurotransmitter acetylcholine at cholinergic synapses and it is one of the fastest enzymes known[65].

Albumin, most known as human serum albumin (HSA), is the most soluble protein in the body (about 4% in serum) and the most prominent protein in plasma. This protein is responsible for a lot of multiple functions in the body, including the maintenance of blood osmolarity, acting as an antioxidant and serving as a solubilising agent and carrier for many endogenous and exogenous compounds[68]. HSA has also the capacity of transporting different substances including fatty acids, hormones, enzymes, dyes, trace metals and drugs. Substances that are toxic in the unbound or free state are generally not toxic when bound to HSA. These properties give to HSA the capability to regulate the extracellular concentration of numerous endogenous as well exogenous administrated substances[69]. HSA does not have an enzyme commission number which means that this protein is considered to be inert, without any catalytic activity. However, it was proven that HSA has esterase activity. The active site of this protein is one tyrosine residue (Tyr411). Although the enzymatic activity of a single molecule of HSA is low, the concentration of this protein is very high, which means that HSA has a significant contribution to drug metabolism[63].

In the human plasma, these four proteins types of esterases are the only ones that have contribution on ester hydrolysis.

1.3.2 Mycobacterial Esterases

As described before, *M. tuberculosis* has a complex cell envelope with mycolic acids, suggesting that there must be a large number of enzymes involved in lipid metabolism[70]. Besides, it was discovered the existence of lipid inclusions in mycobacteria cytoplasm which indicates the presence of lipolytic enzymes[71]. In fact, after the whole genome sequence of this bacteria being studied by the Sanger Center and the *Institut Pasteur*, in 1998, it showed at least 250 enzymes related to lipid metabolism including extracellular secreted enzymes, integrated cell wall enzymes and intracellular lipases/esterases[70]. From the genome annotation of *M. tuberculosis*, it has been concluded that 24 genes encode lipolytic enzymes. These proteins have been classified "Lip family" (LipC to LipZ)[72,73]. However, this classification is only based on the presence of the *consensus* sequence Gly-x-Ser-x-Gly, which is characteristic of the

members of the α/β hydrolase family. This classification does not allow the distinction between lipases and esterases but Blast analysis has shown that the "Lip family" is composed of both lipases and esterases[17]. Esterases preferentially break ester bonds of shorter chain fatty acids and lipases catalyze lipid hydrolysis, which means that lipases display a much broader substrate range than esterases[72]. A fundamental difference between esterases and lipases is their ability to act on solubilised substrates. Esterases act on water-soluble molecules and lipases are highly efficient at hydrolysing molecules aggregated in water, such as an emulsion or a micellar solution[74].

Analysis of the *M. tuberculosis* genome revealed the existence of some carboxylesterases that are responsible for ester hydrolysis. The carboxylesterases identified are members of the "Lip family": Rv3487c (LipF) and Rv1399 (LipH)[75,76]. LipF belongs to the α/β hydrolase-fold family and hydrolyze efficiently short chain esters. The active site of many α/β hydrolases is constituted by a catalytic triad with serine as the nucleophile, histidine as a proton donor and aspartate or glutamate forming the charge-relay network. In LipF, the potential catalytic triad is: Ser90-Glu189-His219, which means that it contains a glutamate residue (Glu) instead of aspartate residue. The aspartate or glutamate residue is strongly bonded to the nitrogen into histidine residue, stabilizing the protonated positively charged protonated, the correct tautomer and the correct conformation of His[76]. Like LipF, LipH belongs to the α/β hydrolase-fold family. This enzyme hydrolyses efficiently short-chain triacylglycerols and vinyl esters and has no detectable activity against emulsified substrates. The catalytic triad of LipH: Ser162-Asp260-His290[75].

In 2010, Guo *et. al* demonstrated that Rv0045c is an esterase of *M. tuberculosis*, involved in ester/lipid metabolism[70]. Compared with LipF and LipH, Rv0045c has shown no sequence identity in a multiple sequence alignment. Although, this alignment showed that the active site (Gly-x-Ser-x-Gly sequence motif) characteristic of hydrolases is highly conserved and the main catalytic residues are Ser89, Asp113, Ser206 and His234, usually present on the catalytic triad of esterases. Like LipF and LipH, Rv0045c can efficiently catalyse short chain ester substrates[70].

1.4 The Aim of the Thesis

In the present work, the main objective was to synthesize nalidixic acid (NAL) derivatives with activity against *M. tuberculosis*. The pretended derivates are long chain esters because they can have an adequate plasma stability and can be activated by mycobacterial esterases[77]. Besides that, NAL is a weak acid and it was previously discovered that *M. tuberculosis* is susceptible to weak acids, suggesting a relation between antimycobacterial activity and p*K*a of acids[78].

So, different nalidixic acid derivatives were synthesized with modifications on carbon C_7 (R_1 = -CH_3 or –CCl_3) and different alkoxyl chains (R_2), as it is shown in **Figure 1.8**:

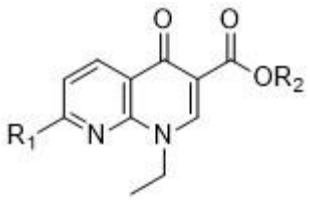


Figure 1. 6 - NAL derivatives representation. R1 = -CH3, -CCl3; R2= different alkoxyl chain.

Stability studies in human plasma and phosphate buffer were also performed in order to evaluate enzymatic and chemical hydrolysis, respectively. The phosphate buffer assays are useful to evaluate if chemical hydrolysis has or not contribution to the velocity of human plasma hydrolysis because it is used a suspension of human plasma 80% in phosphate buffer. Human plasma assays are also useful to evaluate if the synthesized prodrugs are stable and will not degradate until they achieve the target.

Activity studies against *M. tuberculosis* H_{37}Rv strain were done in order to evaluate if compounds inhibit bacterial growth.

With this work, it is pretended to have an ester of nalidixic acid or of a nalidixic acid derivative with slow plasma hydrolysis and activity against *M. tuberculosis.*

Synthesis and Structural Identification

2. Synthesis and Structural Identification

2.1 Ester Synthesis

The esterification reaction is one of the most important and fundamental reactions in organic synthesis[79]. Carboxylic acids react with alcohols to form esters through a condensation reaction (**Figure 2.1**).

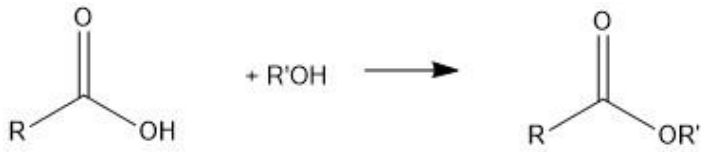


Figure 2. 1 - General reaction of ester synthesis.

Ester synthesis can be achieved in acid-catalysed reactions, that are called Fischer esterifications, or from alkanoyl chlorides (acyl chlorides). In Fischer esterifications (**Figure 2.2**), the carboxylic acid accepts a proton from the strong catalyst. Then the alcohol attacks the protonated carbonyl group to give a tetrahedral intermediate. After that, a proton is lost from one oxygen atom and gained by another one. The loss of a water molecule gives a protonated ester and finally the transfer of that proton to a base leads to the ester[53].

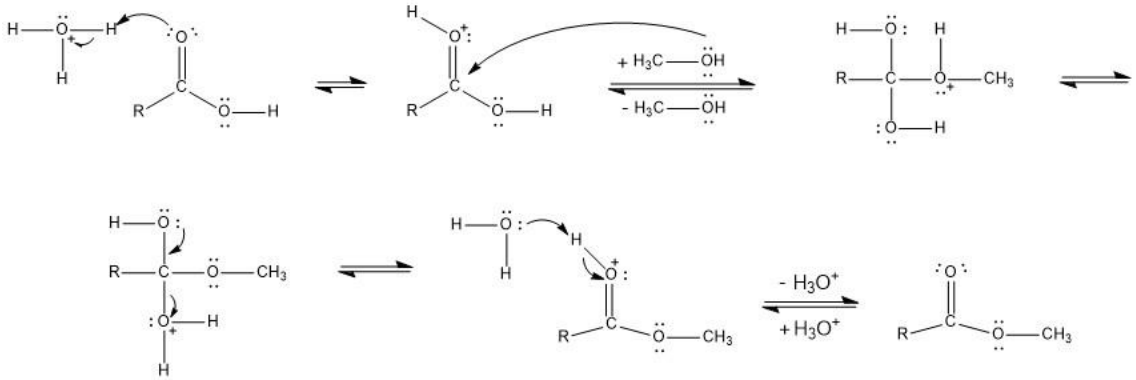


Figure 2. 2 - Mechanism of acid-catalyzed esterification[53].

2.1.1 Esters from Acyl Chlorides

One of the best methods to synthesize esters is the reaction of acyl chlorides with alcohols (**Figure 2.3**). This reaction occurs rapidly and does not require an acid catalyst[53].

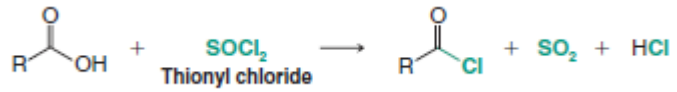


Figure 2. 3 - General reaction of acyl chlorides synthesis with thionyl chloride[53].

Acyl halides are the most reactive of the acyl derivatives; the best synthetic route to an ester is synthesis of an acyl halide, chloride as an example, from the carboxylic acid first and then the conversion of the acyl chloride to the ester, through the reaction with an alcohol.

The reagent used to form acyl chlorides was thionyl chloride because it reacts with carboxylic acids to give acyl chlorides in good yield (**Figure 2.4**):

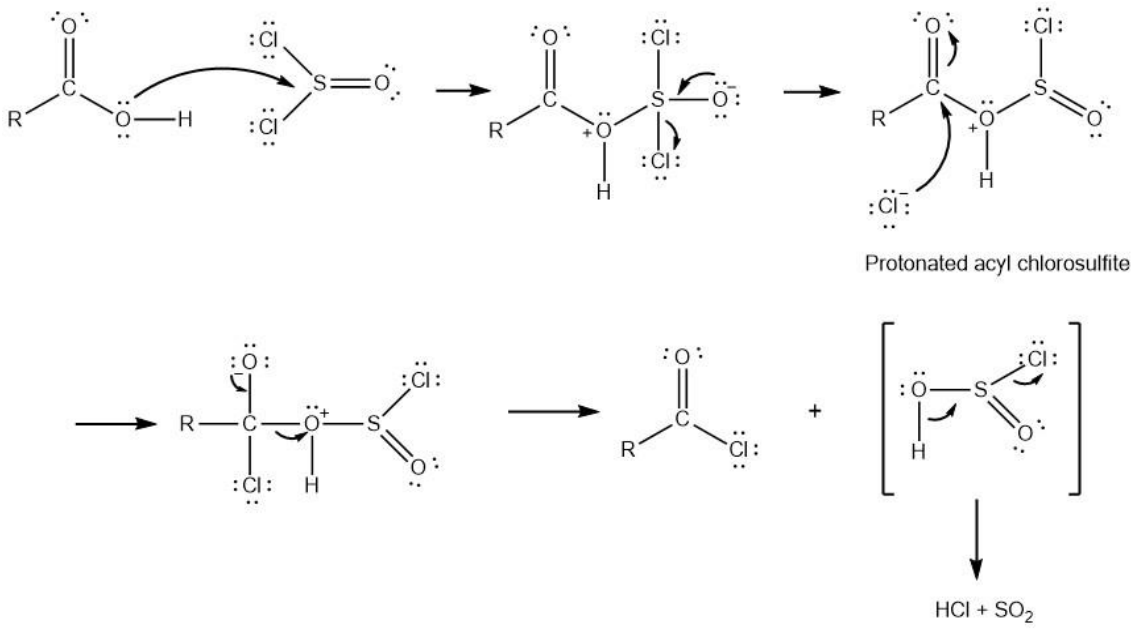


Figure 2. 4 - Mechanism of acyl chlorides synthesis using thionyl chloride[53].

These reactions involve nucleophilic addition-elimination by a chloride ion on a highly reactive intermediate, a protonated acyl chlorosulfite. This intermediate contains even better acyl leaving group than the acyl chloride product[53]

2.2 Synthesis of Nalidixic Acid Esters

In the present work, the main objective was to synthesize two libraries of NAL esters (**Figure 2.5**).

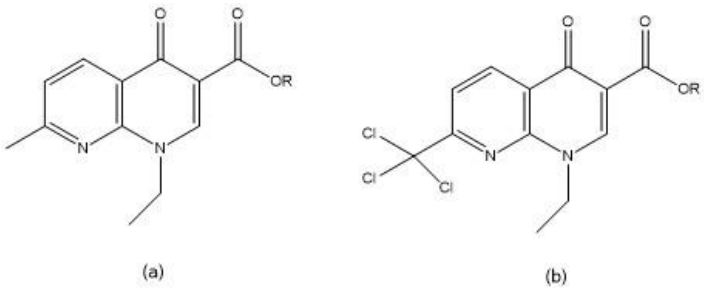


Figure 2. 5 – Libraries of NAL esters. (a) library 1; (b) library 2.

To synthesize the esters of these different libraries, two methods were performed. The first method (method A), was used to synthesize the first library of compounds (**Figure 2.5a**), esters with different alkoxyl chains with the C_7 methyl group on the heteroaromatic acyl group. To synthesize the second esters library, with different alkoxyl chains with the trichloromethyl group on the C_7 heteroaromatic acyl portion (**Figure 2.5b**), method B was used. Method A was based on the description done by Sachdeva *et. al.* in 2015[80] and Method B was based on the description done by Cynamom *et. al.* in 1995[81].

Method A

To 1 equivalent (eq.) of NAL in a round bottom flask, 19.2 eq. of thionyl chloride were added and the flask was kept in fuming chamber aside for 15 min. Later 34.4 eq. of the alcohol were added to the solution drop by drop and mixed thoroughly after each addition. Then the reaction was kept under reflux during 1 hour (h) (**Figure 2.6**)[80].

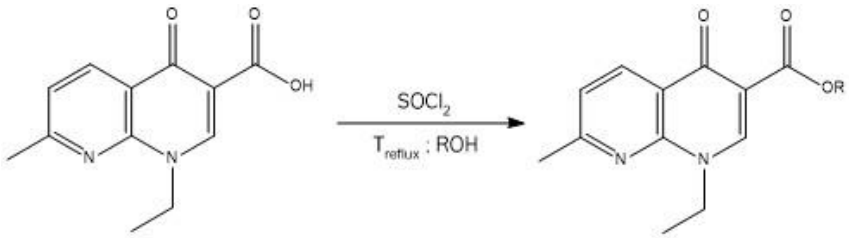


Figure 2. 6 – Scheme of ester synthesis by method A.

Method B

To 1.2 eq. of NAL in a round bottom flask, 16.5 eq. of thionyl chloride were added and this reaction was kept under reflux for 2h. The acyl chloride was isolated as solid residue by evaporation of the thionyl chloride in excess under vacuum on a rotary evaporator. Meanwhile, 1.5 eq. of NaH were added to 1 eq. of alcohol to prepare its alkoxide. Then, the alkoxide was added to the acyl chloride solid residue previously dissolved in dried dichloromethane and this reaction was kept under reflux to synthesize the ester (**Figure 2.7**). The reaction was followed by thin layer chromatography.

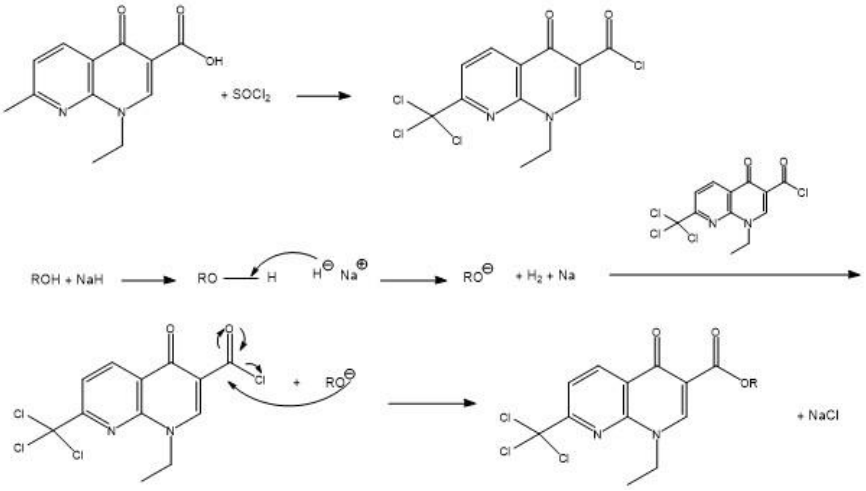
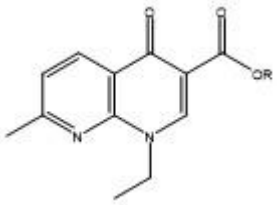


Figure 2. 7 - Scheme of ester synthesis by method B.

2.2.1 First library of Esters

The first esters library was synthesized by method A. These esters have different alkoxyl chain, ranging from 2 until 14 carbons length.

Table 2. 1 - First library of NAL esters.



	R	Reflux (h)	Yield (%)	Description
2	C_2H_5	1	65	White solid
3	C_4H_9	1	68	White solid
4	C_6H_{13}	1	59	White solid
5	C_9H_{19}	1	51	White solid
6	$C_{10}H_{21}$	1	60	White solid
7	$C_{11}H_{23}$	1	65	White solid
8	$C_{12}H_{25}$	1	66	Yellow solid
9	$C_{14}H_{29}$	1	63	Yellow solid
10	$CH(CH_3)C_7H_{15}$	1	79	Yellow solid

In the first library of compounds, 9 different esters were synthesized with different alkoxyl chains from 2 to 14 carbons length and they were obtained with a range of yields from 51% to 79%. The esters **2-9**

have linear alkoxyl chains and the ester 10 has a linear alkoxyl chain of 8 carbons with one ramification (methyl group) in the first carbon of the chain (**figure 2.8**).

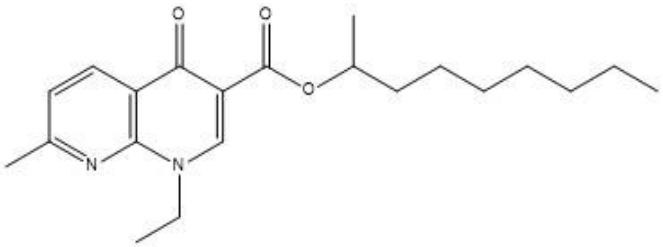
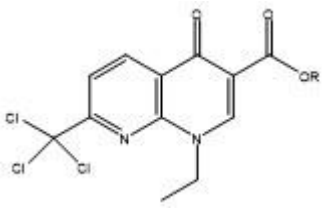


Figure 2. 8 – Structure of compound 10.

2.2.2 Seconds Library of Esters

The second esters library was synthesized by method B. These esters have different alkoxyl chains, from 4 until 14 carbons length.

Table 2. 2 - Second library of NAL derivatives.



	R	Reflux (h)	Yield (%)	Description
1a	H	overnight	100	Brown solid
3a	C_4H_9	18	21	White solid
5a	C_9H_{19}	19	5	White solid
6a	$C_{10}H_{21}$	11	5	Yellow solid
7a	$C_{11}H_{23}$	11	15	Yellow solid
8a	$C_{12}H_{25}$	40	15	Yellow solid
9a	$C_{14}H_{29}$	16	35	Yellow solid
10a	$CH(CH_3)C_7H_{15}$	33	20	Yellow oil

In the second ester library group, 7 different esters were synthesized and the yields obtained were very low, ranging from 5% to 35%. This may be due to the excess of acid in this method instead the alkoxide (like in method A, but with the alchohol).

This method starts with 2h of reflux of the acid with thionyl chloride. This step led to the synthesis of the acyl chloride and also the chlorination of the methyl group in carbon C_7 of the aromatic acyl group. This substitution occurs because chlorination occurs rapidly in methyl groups attached *ortho* or *para* to a nitrogen atom in a multi-heteroatom ring[82].

Compound 1a is not an ester, is a carboxylic acid derivative of nalidixic acid. This compound was synthesized as a control for the stability assays. Like the synthesis of the esters of this library, nalidixic acid was kept under reflux with thionyl chloride for 2 hours. Then, the excess of thionyl chloride was evaporated and water was added until the carboxylic acid was formed.

The esters obtained through this method have linear alkoxyl chains except the compound **10a** which has a linear alkoxyl chain of 8 carbons with one ramification on the first carbon of the chain (**Figure 2.9**).

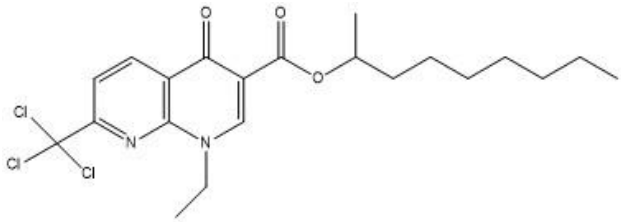


Figure 2. 9 - Structure of compound 10a.

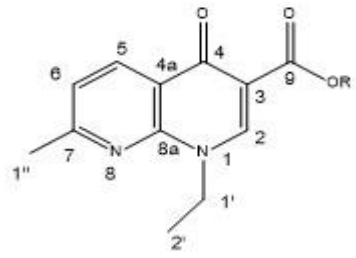
2.3 Structural Identification of Synthetized Esters

2.3.1 Nuclear Molecular Ressonance

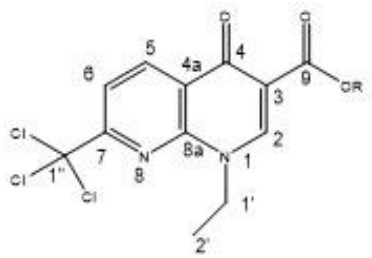
The proton (^1H) and carbon (^{13}C) NMR spectra were obtained in deuterated chloroform ($CDCl_3$). All the NMR spectra of the synthesized compounds are characterized in chapter 7.

All the signals identification have been made with the help of Heteronuclear Multiple-Quantum Correlation (HMQC) and Heteronuclear Multiple Bond Correlation (HMBC) and Attached Proton Test (APT) spectra for carbons besides the normal proton (^1H) and carbon (^{13}C) spectra, as can be seen an example in Appendix 1.

Table 2. 3 - ^{1}H-NMR signal (ppm) of aromatic protons and protons attached to $C_{1'}$, $C_{2'}$ and $C_{1''}$ in libraries 1 and 2.



Library 1



Library 2

Compound Library 1	Aromatic protons	$C_{1'}$-*H*	$C_{2'}$-*H*	$C_{1''}$-*H*

1	8.91 (C_2-*H*); 8.67 (C_5-*H*); 7.41 (C_6-*H*)	4.63	1.55	2.75
2	8.65 (C_5-*H*); 8.63 (C_2-*H*); 7.24 (C_6-*H*)	4.49	1.50	2.66
3	8.63 (C_5-*H*); 8.60 (C_2-*H*); 7.23 (C_6-*H*)	4.48	1.48	2.65
4	8.57 (C_5-*H*); 8.54 (C_2-*H*); 7.16 (C_6-*H*)	4.41	1.42	2.59
5	8.57 (C_5-H); 8.53 (C_2-H); 7.16 (C_6-H)	4.41	1.42	2.58
6	8.65 (C_5-*H*); 8.61 (C_2-*H*); 7.24 (C_6-*H*)	4.33	1.49	2.66
7	8.65 (C_5-*H*); 8.61 (C_2-*H*); 7.24 (C_6-*H*)	4.48	1.49	2.66
8	8.65 (C_5-*H*); 8.61 (C_2-*H*); 7.24 (C_6-*H*)	4.48	1.49	2.66
9	8.57 (C_5-*H*); 8.54 (C_2-*H*); 7.17 (C_6-*H*)	4.41	1.42	2.59

Compound Library 1	**Aromatic protons**	**$C_{1'}$-*H***	**$C_{2'}$-*H***	**$C_{1''}$-*H***
10	8.64 (C_5-*H*); 8.57 (C_2-*H*); 7.23 (C_6-*H*)	4.48	1.49	2.65
Compound Library 2	**Aromatic protons**	**$C_{1'}$-*H***	**$C_{2'}$-*H***	**$C_{1''}$-*H***
1a	9.03 (C_2-*H*), 8.99 (C_5-*H*); 8.22 (C_6-*H*)	4.67	1.63	-
3a	8.92 (C_5-*H*); 8.70 (C_2-*H*); 8.06 (C_6-*H*)	4.52	1.57	-
5a	8.93 (C_5-*H*); 8.71 (C_2-*H*); 8.06 (C_6-*H*)	4.53	1.58	-
6a	8.93 (C_5-*H*); 8.71 (C_2-*H*); 8.07 (C_6-*H*)	4.36	1.58	-
7a	8.92 (C_5-*H*); 8.69 (C_2-*H*); 8.06 (C_6-*H*)	4.52	1.57	-
8a	8.93 (C_5-*H*); 8.71 (C_2-*H*); 8.07 (C_6-*H*)	4.53	1.58	-
9a	8.84 (C_5-*H*); 8.62 (C_2-*H*); 7.98 (C_6-*H*)	4.44	1.49	-
10a	8.91 (C_5-*H*); 8.66 (C_2-*H*); 8.05 (C_6-H)	4.52	1.58	-

After ^{1}H-NMR spectra analysis, it is observed that chemical shifts of aromatic protons are different between the two libraries of compounds staying in general at higher positions in the second library (**table 2.3**). In the first library, C_5-*H* protons have a chemical shift between 8.57-8.65 ppm, C_2-*H* between 8.53-8.63 ppm and C_6-*H* between 7.16-7.24 ppm and in the second library, C_5-*H* protons have a chemical shift between 8.93-8.84 ppm, C_2-*H* between 8.71-8.62 ppm and C_6-*H* between 8.07-7.98 ppm. These differences occur due to the substitution of the methyl group hydrogens for three chlorines. The same fact is also observed in proton spectra of the two acids: **1** with C_7-methyl and **1a** with C_7-chloromethyl groups.

Also, when comparing each group of esters with their acid (**table 2.3**) it is possible to conclude that after ester formation, all the ^{1}H-NMR signals of the spectra for both groups of esters have a lower chemical shift more evident in the signal of C_2-*H* and an exchange between the positions of C_2-*H* and C_5-*H* signals is observed from the acid to the esters.

Other difference between the ^{1}H-NMR spectra of the two libraries is the existence of a singlet at 2.58-2.66 ppm on library 1 which corresponds to the methyl group attached to carbon C_7. This singlet signal does not exist in compounds of library 2 because the protons of the methyl group attached to carbon C_7 were substituted by three chloro atoms on the trichloromethyl group.

Compounds **10** and **10a** with a methyl group connected to carbon $C_{1'''}$ of the alkoxyl chain transforms this carbon in a *chiral* carbon, that is an asymmetric atom or stereocenter, and this fact implies that the two protons of the next carbon on the alkoxyl chain $C_{2'''}$ are nonequivalent and are called diastereotopic, as such two different signals appear in the proton ^{1}H-NMR spectra. Therefore instead of the signal $C_{2'''}$-*H* of the two equivalent protons, like on the other esters, due to the splitting with $C_{1'''}$ and $C_{3'''}$ protons resulting in a *tt* which appears as a multiplet with two protons integration, these signals will result in a *ddt* splitting appearing as two multiplet signals with one proton integration each.

The aromatic protons C_5-*H* and C_6-*H* appear as duplets and the coupling constants (*J*) were:

$J_{5\text{-}6}$ = 8.1 Hz (library 1),

$J_{5\text{-}6}$ = 8.1-8.4 Hz (library 2),

With the ethyl group N_1-$C_{1'}H_2$-$C_{2'}H_3$ the $C_{1'}$-*H* appears as a quadruplet and $C_{2'}$-*H* as a triplet with a coupling constant:

$JC_{1'}H$-$C_{2'}H$ = 7.2 Hz (both groups of compounds).

On the other hand with the alkoxyl protons the chains last carbon appears as a triplet with coupling constants:

$J_{(\text{last carbon-}H)} \approx$ 6.9-7.8 Hz.

And for both groups of compounds, except **10** and **10a**, $C_{1'''}$-*H* appears as a triplet with coupling constants:

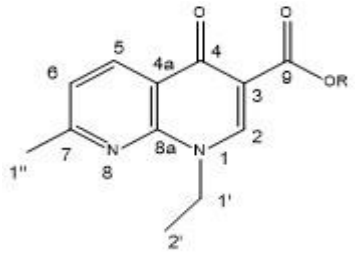
$J\ C_{1'''}$-*H*' ≈ 6.6-7.2 Hz

Whereas in compounds **10** and **10a** the protons CH_3-$C_{1'''}$appear as a duplet with a coupling constant:

$J\ CH_3$-$C_{1'''}$-H = 6.3 Hz (compounds 10 and 10a).

The protons in the alkoxyl chains have an overlap in the protons signal being more common in the longer alkoxyl chains.

Table 2. 4 - ^{13}C-NMR signal (ppm) of carbons in libraries 1 and 2.



Library 1

Compounds Library 1	Aromatic carbons	C_9	$C_{1'}$	$C_{2'}$	$C_{1''}$
2	174.74 (C_4); 162.66 (C_7); 148.74 (C_2); 148.58 (C_{8a}); 136.86 (C_5); 121.51 (C_{4a}); 121.14 (C_6); 112.48 (C_3)	165.64	46.53	15.03	24.96
3	174.71 (C_4); 162.62 (C_7); 148.68 (C_2); 148.60 (C_{8a}); 136.86 (C_5); 121.52 (C_{4a}); 121.10 (C_6); 112.06 (C_3)	165.77	46.57	15.22	25.08
4	174.74 (C_4); 162.63 (C_7); 148.66 (C_2); 148.62 (C_{8a}); 136.89 (C_5); 121.53 (C_{4a}); 121.11 (C_6); 112.06 (C_3)	165.72	46.57	15.21	25.08

Compounds Library 1	Aromatic carbons	C_9	$C_{1'}$	$C_{2'}$	$C_{1''}$
5	174.73 (C_4); 162.62 (C_7); 148.66 (C_2); 148.61 (C_{8a}); 136.89 (C_5); 121.54 (C_{4a}); 121.10 (C_6); 112.07 (C_3)	165.72	46.57	15.21	25.08
6	174.72 (C_4); 162.62 (C_7); 148.66 (C_2); 148.62 (C_{8a}); 136.89 (C_5); 121.54 (C_{4a}); 121.10 (C_6); 112.07 (C_3)	165.73	46.57	15.21	25.08
7	174.71 (C_4); 162.61 (C_7); 148.66 (C_2); 148.62 (C_{8a}); 136.89 (C_5); 121.55 (C_{4a}); 121.09 (C_6); 112.09 (C_3)	165.74	46.56	15.22	25.08
8	174.73 (C_4); 162.63 (C_7); 148.66 (C_2); 148.61 (C_{8a}); 136.89 (C_5); 121.54 (C_{4a}); 121.11 (C_6); 112.07 (C_3)	165.72	46.57	15.22	25.08
9	174.74 (C_4); 162.63 (C_7); 148.66 (C_2); 148.61 (C_{8a}); 136.89 (C_5); 121.53 (C_{4a}); 121.11 (C_6); 112.07 (C_3)	165.72	46.57	15.21	25.08
10	174.76 (C_4); 162.54 (C_7); 148.62 (C_2); 148.37 (C_{8a}); 136.87 (C_5); 121.52 (C_{4a}); 121.02 (C_6); 112.49 (C_3)	165.07	46.52	15.19	25.06
Compounds Library 2	**Aromatic carbons**	**C_9**	**$C_{1'}$**	**$C_{2'}$**	**$C_{1''}$**
1a	178.04 (C_4); 161.52 (C_7); 155.21 (C_2); 149.73 (C_{8a}); 147.44 (C_5); 139.16 (C_{4a}); 121.89 (C_6); 110.82 (C_3);	165.85	48.56	15.09	117.84

3a	173.69 (C_4); 160.02 (C_7); 149.78 (C_2); 147.48 (C_{8a}); 139.55 (C_5); 124.08 (C_{4a}); 116.59 (C_6); 113.07 (C_3)	165.18	47.64	14.98	96.39
5a	171.71 (C_4); 160.03 (C_7); 149.74 (C_2); 147.48 (C_{8a}); 139.57 (C_5); 124.09 (C_{4a}); 116.58 (C_6); 113.09 (C_3)	165.15	47.62	14.96	96.40
6a	173.74 (C_4); 160.01 (C_7); 149.77 (C_2); 147.52 (C_{8a}); 139.59 (C_5); 124.10 (C_{4a}); 116.57 (C_6); 113.09 (C_3);	165.19	47.63	14.97	96.44
7a	173.72 (C_4); 160.03 (C_7); 149.68 (C_2); 147.49 (C_{8a}); 139.56 (C_5); 124.09 (C_{4a}); 116.55 (C_6); 113.06 (C_3)	165.73	47.65	14.96	96.42
8a	173.86 (C_4); 159.99 (C_7); 149.59 (C_2); 147.65 (C_{8a}); 139.89 (C_5); 124.11 (C_{4a}); 116.58 (C_6); 113.12 (C_3)	165.77	47.63	14.94	96.35
9a	173.69 (C_4); 160.03 (C_7); 149.75 (C_2); 147.49 (C_{8a}); 139.57 (C_5); 124.10 (C_{4a}); 116.57 (C_6); 113.10 (C_3)	165.15	47.61	14.96	96.41
10a	173.64 (C_4); 160.02 (C_7); 149.72 (C_2); 147.51 (C_{8a}); 139.60 (C_5); 124.14 (C_{4a}); 116.57 (C_6); 113.11 (C_3)	165.16	47.52	14.93	96.39

In ^{13}C-NMR spectra analysis (**table 2.4**), the signal of the carbonyl carbon (C_4) appears at 171-178 ppm and the aromatic carbons at: 159-162 ppm (C_7), 148-155 ppm (C_2), 147-149 ppm (C_{8a}), 136-147 ppm (C_5), 121-139 ppm (C_{4a}), 116-121 ppm (C_6) and 110-113 ppm (C_3). The signal of carbon C_9 appears at 165 ppm. The carbon linked to the nitrogen atom ($C_{1'}$) has a chemical shift of 46-48 ppm and the other carbon of this ethyl group ($C_{2'}$) has a chemical shift of 14-15 ppm. On the alkoxyl chain, the carbon with lower chemical shift is the last one of the chain that is 13-14 ppm. On the other hand, the carbon with higher chemical shift in the alkoxyl chain ($C_{1'''}$) is the one linked to the oxygen of the ester group that has 60-71 ppm. Compounds **10** and **10a** display a signal at 71 ppm for carbon $C_{1'''}$ since this carbon becomes tertiary in these compounds due to the methyl substitution instead of being secondary carbon like in the other esters with linear alkoxyl chains, and this fact leads to a higher chemical shift.

These results show that between the two libraries of compounds there are no significant differences between the chemical shifts of aromatic carbons and carbons of the ethyl group linked to the nitrogen. The main difference in ^{13}C-NMR spectra of the two libraries is the signal of carbon $C_{1''}$. On library 1, this carbon has a chemical shift 25 ppm and on library 2 of 96 ppm. This happens because on library one this carbon is linked to a methyl group but on library 2 is linked to a trichloromethyl group. Since the electronegativity of the chloro atom is higher than that of the hydrogen atom, the $C_{1''}$ signal on library 2 appears at a much higher chemical shift.

2.3.2 Mass Spectrometry

To confirm the structure of compounds of library 2, mass spectromety analysis of compound 9a was performed (**Figure 2.10**)

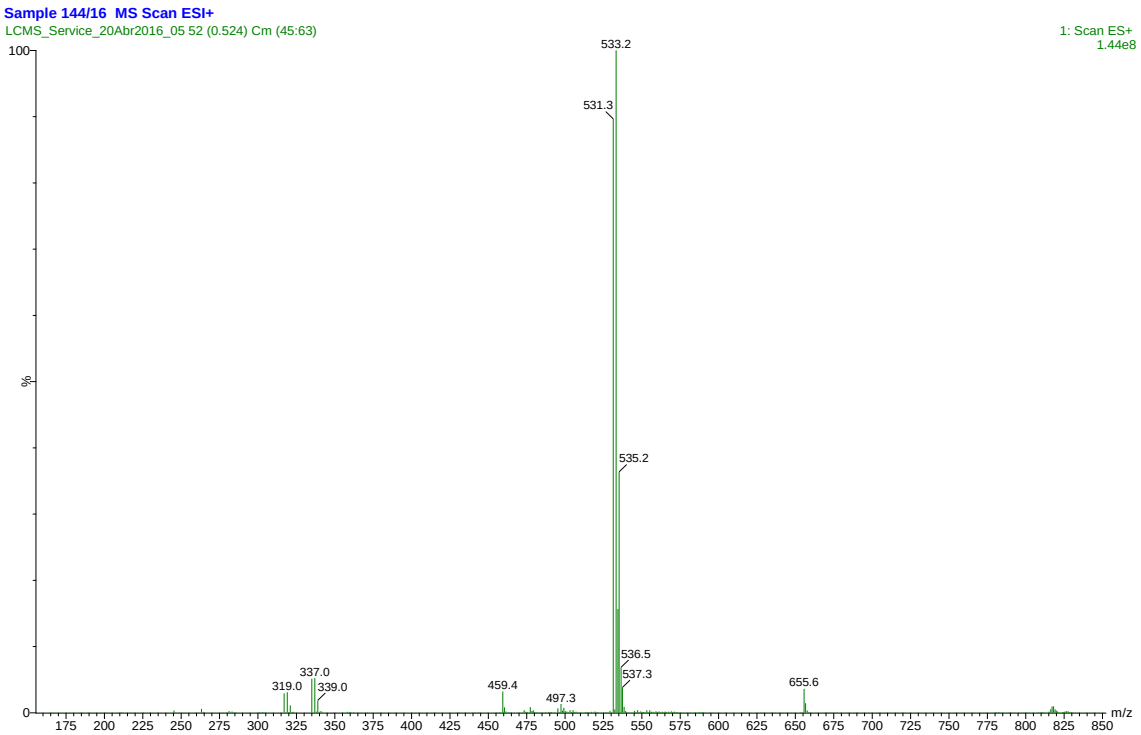


Figure 2. 10 - Mass spectrometry result of compound 9a.

As it is possible to observe, the base peak is the peak with a m/z of 533.2, so it is the most intense ion with an abundance of 100%. Since the molecular weight of the compound **9a** is 531.9, this peak represents the gain of a proton[83].

The most important result of this analysis is the confirmation of chlorine presence of the structure. Chlorine is composed by two isotopes ^{35}Cl and ^{37}Cl[83].

Since there are 3 chlorine atoms on the molecule, there are 8 possibles was to combine the isotopes. The only way these molecules can be distinguished is by their m/z values and there are 4 possible values: 531.3, 533.2, 535.2 and 537.3. So with these results, it is possible to confirm the substitution of the methyl group in carbon C_7 by a trichloromethyl group in compounds of library 2.

Stability and Activity Assays

3. Stability and Activity Assays

Chemical stability of the synthesized esters was studied in phosphate buffer pH=7.4 and human plasma. These assays are very important to evaluate the stability of all the synthesized compounds to hydrolysis (chemical and enzymatic) before they reach the mycobacteria. Hydrolysis of the esters was analysed through high performance liquid chromatography (HPLC) and it was observed the evolution with the time of ester (substrate) and acid (hydrolysis product) concentration. These studies have been performed as it's described in chapter 7.

The activity assays *in vitro* were performed by Nuno Carmo PhD from Host-Pathogen Interactions group of iMed ULisboa, led by Professor Elsa Anes.

3.1 Stability Assays in Phosphate Buffer

Chemical hydrolysis of esters was studied in phosphate buffer pH = 7.4 in order to evaluate the interference of this hydrolysis on the enzymatic assays. Phosphate buffer solution was prepared with a total concentration of 0.05 M, ionic strength 0.15 M and pH = 7.4. Chemical stability of all esters was evaluated in this pH in solutions with 2% of acetonitrile and at temperature of 37ºC - the pH and temperature of human body. Ester hydrolysis result on the appearance of the respective acid.

In these assays, phosphate buffer pH 7.4 had a total concentration of 0.05M, ionic strength of 0.15, 2% of acetonitrile (ACN) and the initial concentration of substrates was $5x10^{-5}$M. To all the assays performed, *pseudo*-first order constants (k_{obs}) and half-time lives ($t_{1/2}$) were obtained as an estimation since the observed ester degradation was quite low and the necessary 3 $t_{1/2}$ to obtain a good decay profile were never achieved. To compare these results with human plasma assays, it was also predicted the degradation in 72h of assay (time of human plasma assays). Those estimated results are shown on the table below (**table 3.1**).

Table 3. 1 – Hydrolysis of compounds of libraries 1 and 2 in phosphate buffer pH=7.4.

(Ct= 0.05M, I=0.15M with 2% of ACN) pH=7.4, T=37ºC, n=2 – number of samples for each compound. Initial concentration of substrate= $5x10^{-5}$M.

Compound Library 1	**$(10^{-3}x)k_{obs}/h^{-1}$**	**$t_{1/2}$/day**	**Degradation**	**Degradation (% in 72h)**
2	0.31	92.7	8% in 356h	1.6
3	0.35	82.4	9% in 356h	1.8
4	0.42	67.4	11% in 356h	2.2
5	1.1	26.3	14% in 177h	5.7
6	1.7	16.8	22% in 177h	8.9
7	0.8	33.7	11% in 178h	4.4
8	2.1	13.8	31% in 157h	14.2
9	3.6	8.0	52% in 207h	18.1
10	0.9	33.3	13% in 208h	4.5
Compound Library 2	**$(10^{-3}x)k_{obs}/h^{-1}$**	**$t_{1/2}$/day**	**Degradation**	**Degradation (% in 72h)**
3a	2.0	14.1	53% in 359h	10.6
5a	3.6	7.8	44% in 165h	19.2
6a	0.8	36.1	21% in 288h	5.3

Compound Library 2	(10^{-3}x)k_{obs}/h^{-1}	$t_{1/2}$/day	Degradation	Degradation (% in 72h)
7a	0.7	37.3	9% in 161h	4.0
8a	1.1	26.4	13% in 165h	5.7
9a	1.7	16.9	22% in 167h	9.5
10a	0.8	38.2	9% in 165h	3.9

In these assays it is possible to observe that k_{obs} vary between $3.6x10^{-3}$ h^{-1} (compound **9** and **5a**) and $0.31x10^{-3}$ h^{-1} (compound **2**). With these results it is possible to conclude that compound **2** is the most stable to chemical hydrolysis at pH 7.4 and at the temperature of 37ºC. It is also possible to conclude that compounds of library 1 are, in general, more stable than compounds of library 2. These studies also show that all the esters are very stable to chemical hydrolysis because all have long estimated half-time lives. The most unstable compound, **5a**, has a $t_{1/2}$ of 7.8 days which is a high $t_{1/2}$.

With these stability studies in phosphate buffer at pH 7.4 and T=37ºC, it is possible to observe that the disappearance of the ester corresponds to the acid formation (**figures 3.1** and **3.2**). These results were obtained in duplicate assays and the concentrations were calculated based on the calibration curves of each compound.

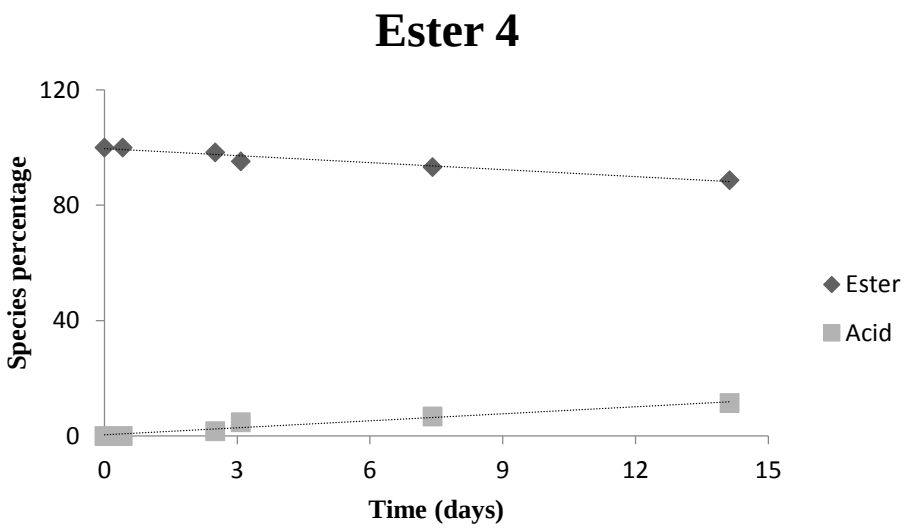


Figure 3. 1 – Variation of ester and acid concentration during phosphate buffer (pH=7.4, T=37ºC) assay of ester 4.

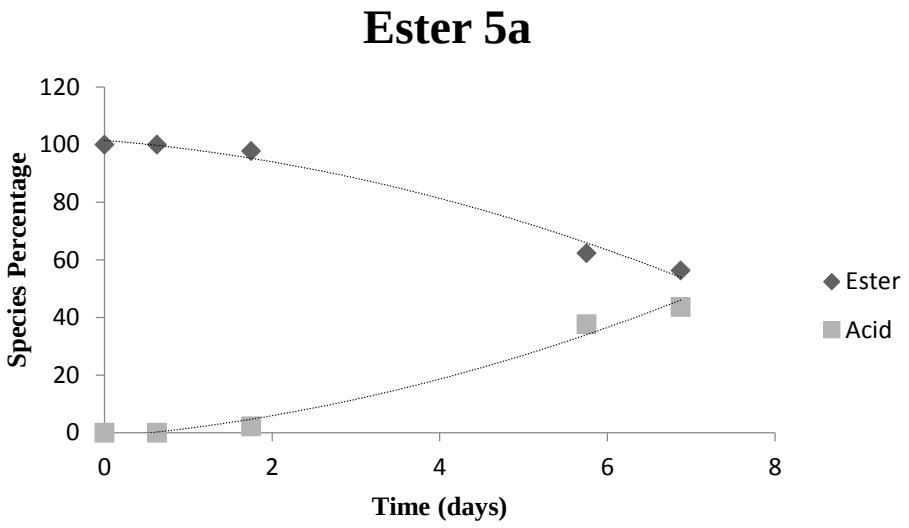


Figure 3. 2 - Variation of ester and acid concentration during phosphate buffer (pH=7.4, T=37ºC) assay of ester 5a.

On **figure 3.1**, it is possible to observe that compound **4** is very stable on phosphate buffer pH 7.4 at the temperature 37ºC since there is almost no degradation of the ester and then no formation of the correspondent acid. These results show that ester **4** is very stable to chemical hydrolysis, in those conditions of pH and temperature.

With ester **5a** in phosphate buffer – pH 7.4 and temperature 37ºC (**figure 3.2**) it is possible to observe the degradation of compound **5a** and the formation of the correspondent acid. This compound is also very stable to chemical hydrolysis because his $t_{1/2}$ is high (7.8 days) but is less stable than compound **4** in the same conditions of pH and temperature.

3.2 Stability Assays in Human Plasma

To evaluate enzymatic hydrolysis, stability assays in human plasma were performed. The desirable result is that esters have low rates of hydrolysis in order to reach *M. tuberculosis* with good concentration and to be hydrolysed there by mycobacterial esterases.

In these assays, 80% of human plasma was diluted in phosphate buffer pH 7.4 with total concentration of 0.05M, ionic strength of 0.15M, 2% of ACN and the initial concentration of substrates was $5x10^{-4}$M. Like with the phosphate buffer assays, in human plasma assays (k_{obs}) and $t_{1/2}$ were obtained as an estimation since the observed ester degradation was quite low and the 3 half-time lives could never be achieved within the established 72h the plasma stays active. Therefore, those estimates were evaluated to all the assays and are presented on the **table 3.2**.

Table 3. 2 - Hydrolysis of compounds of libraries 1 and 2 in 80% of human plasma suspension in phosphate buffer pH=7.4.

(Ct= 0.05M, I=0.15M with 2% of ACN) pH=7.4, T=37ºC, n=3 – number of samples for each compound. Initial concentration of substrate= $5x10^{-4}$M.

Compound Library 1	**(10^{-3}x)k_{obs}/h^{-1}**	**$t_{1/2}$/day**	**Degradation (% in 72h)**
2	1.2	25	6
3	nd	nd	nd
4	nd	nd	nd
5	1.2	25	6
6	nd	nd	nd
7	2.9	10	15
8	nd	nd	nd
9	nd	nd	nd
10	nd	nd	nd
Compound Library 2	**(10^{-3}x)k_{obs}/h^{-1})**	**$t_{1/2}$/day**	**Degradation (% in 72h)**
3a	3.1	9.4	16
5a	10.2	2.8	53
6a	1.2	24.2	6.2
7a	nd	nd	nd
8a	8.3	3.5	13
9a	nd	nd	nd
10a	nd	nd	nd

nd – no visible degradation within the 72h of the plasma assays.

The estimated results for the stability in human plasma show that all the compounds are very stable. The k_{obs} vary between $10.2x10^{-3}$ h^{-1} (compound **5a**) and $0.2x10^{-3}$ h^{-1} (compound **7**) for the esters that have shown degradation. Like in phosphate buffer assays, compound **5a** was the most susceptible for hydrolysis. For most of the esters it was not possible to calculate the k_{obs} and the $t_{1/2}$ because in 72h assay these esters did not hydrolyse at all. So, these results show that all the synthesized compounds are stable in human plasma.

In these stability studies in 80% human plasma in phosphate buffer at pH 7.4, it is possible to observe that the disappearance of the ester corresponds to the acid formation, for those esters that suffered hydrolysis (**figures 3.3 and 3.4**). These results were obtained in triplicate assays and the concentrations along the assays were calculated with calibration curves of each compound.

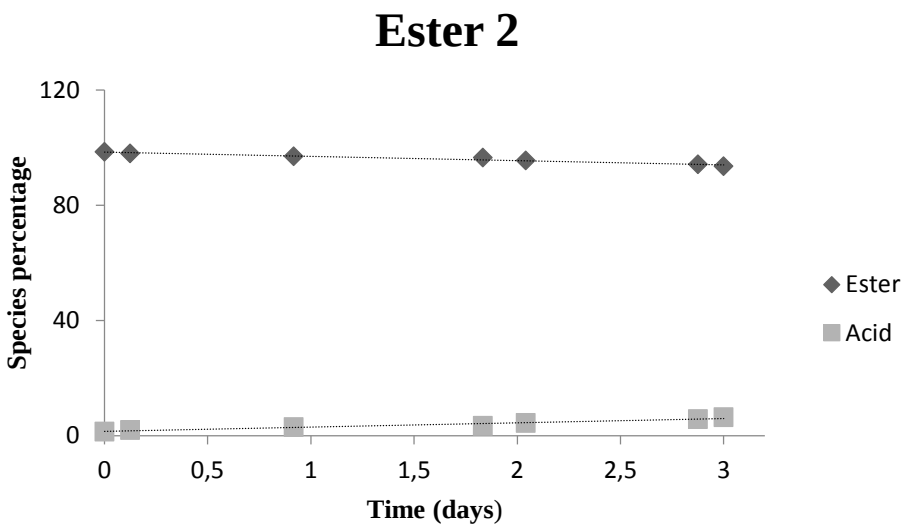


Figure 3. 3 - Variation of ester and acid concentration during human plasma assay of ester 2.

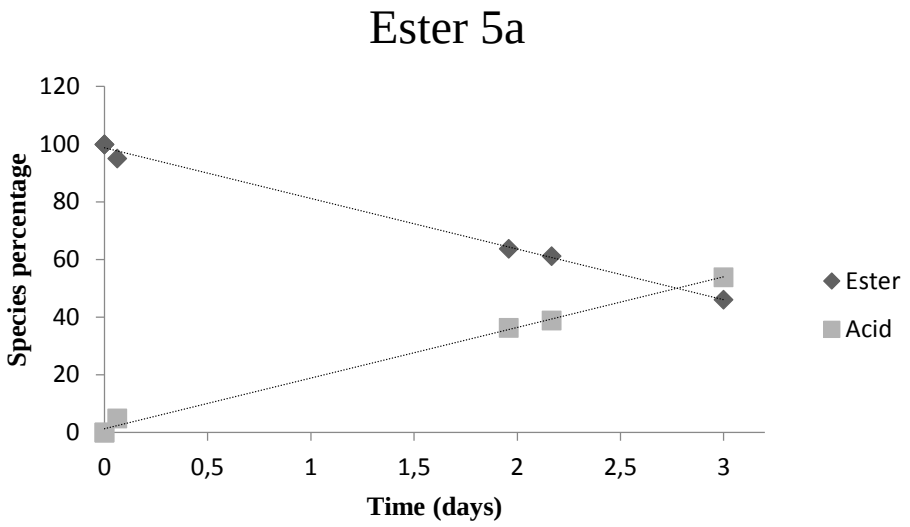


Figure 3. 4 - Variation of ester and acid concentration during human plasma assay of ester 5a.

On **figure 3.3**, it is possible to observe that compound **2** is very stable in human plasma assays since there is almost no degradation of the ester and so no formation of the correspondent acid. These results show that ester **2** is very stable to human plasma enzymatic hydrolysis.

On the other hand, it is possible to observe the degradation of compound **5a** and the formation of the correspondent acid (**figure 3.4**). It is possible to conclude that this compound is also very stable to human plasma enzymatic hydrolysis because his $t_{1/2}$ is high (2.8 days) even being less stable than compound **2**.

3.3 Ratio between Human Plasma Stability/Phosphate Buffer pH 7.4 Stability

To evaluate the contribution of chemical hydrolysis in human plasma enzymatic hydrolysis, *pseudo*-first order constants obtained in phosphate buffer pH 7.4 assays were compared with those obtained in human plasma assays (enzymatic hydrolysis) – **table 3.3**.

Table 3. 3 – Ration between $(10^3x)k_{obs}/(h^{-1})$ of plasma hydrolysis and $(10^3x)k_{obs}/(h^{-1})$ of phosphate buffer pH 7.4 hydrolysis.

Compound Library 1	$(10^3x)k_{obs}/h^{-1}$ Human plasma	$(10^3x)k_{obs}/h^{-1}$ Phosphate buffer	Ratio (k_{plasma}/k_{buffer})
2	1.2	0.31	4
3	nd	0.35	-
4	nd	0.42	-
5	1.2	1.1	1.1
6	nd	1.7	-
7	2.9	0.8	3.6
8	nd	2.1	-
9	nd	3.6	-
10	nd	0.9	-
Compound Library 2	$(10^3x)k_{obs}/h^{-1}$ Human plasma	$(10^3x)k_{obs}/h^{-1}$ Phosphate buffer	**Ratio**
3a	3.1	2.0	1.6
5a	10.2	3.6	2.8
6a	1.2	0.8	1.5
7a	nd	0.7	-
8a	8.3	1.1	7.5
9a	nd	1.7	-
10a	nd	0.8	-

nd – no visible degradation within the 72h of the plasma assays.

For some of the esters it was not possible to calculate the ratio between enzymatic hydrolysis (human plasma assays) and chemical hydrolysis (phosphate buffer pH 7.4 assays) because in plasma assays these compounds have a small degradation during the 72h – the time of the plasma assays. For compounds **2**, **5**, **7**, **3a**, **5a**, **6a** and **8a** it was possible to calculate the ratio because they have a little degradation within the 72h of human plasma assays. With these results it is possible to conclude that on esters **2**, **7, 5a** and **8a** k_{obs} in human plasma enzymatic hydrolysis is higher than k_{obs} of chemical hydrolysis, since the ratio values are 4, 3.6, 2.8 and 7.5, respectively. On the other esters (**5**, **3a** and **6a**), the results indicate that both k_{obs} of human plasma enzymatic hydrolysis and chemical hydrolysis are very similar. It is possible to conclude this because the ratios obtained for these compounds are approximately 1.

For compounds that was not possible to calculate the ratios (**3**, **4**, **6**, **8**, **9**, **10**, **7a**, **9a** and **10a**) are very stable to human plasma enzymatic hydrolysis because they have shown no degradation during the 72h of assay and so it was not possible to estimate a k_{obs} and a $t_{1/2}$.

3.4 Activity Studies *in vitro*

Ester activities were studied in *M. tuberculosis* H_{37}Rv strain, a virulent strain derived from the parent strain H_{37}, that was primarily isolated from a patient with chronic pulmonary TB[84]. The results obtained are shown in the table below (**Table 3.4**) and the activities are presented in minimum inhibitory concentration (MIC_{90}) in µM, which represents at least 90% of inhibition of the bacterial growth.

Samples stock solutions were prepared in ethanol with a concentration of 10^{-2}M and dilutions 1:100 were performed to 96-well microplates (clear flat bottom) at a final volume of 200µL per well. The final concentrations in the 96-well microplates were in the range 100µM to 3.125 µM. All the compounds were tested against the recombinant *M. tuberculosis* H_{37}Rv (pMV306hsp + LuxG13) provided by MTA Addgene. The medium used was 7H9 supplemented with OADC (oleic acid, albumin, dextrose and catalase). The assays were done for 5 days, at neutral pH, T=37ºC in a BSL3 incubator with 5% of CO_2. The wavelength used to measure the optical densities was 600nm[85–87]. The MIC_{90} was defined as the lowest concentration of the compound effecting a reduction of 90% in absorbance relative to the controls, and the 50% inhibition the minimum concentration where a reduction in absorbance above 50% was observed.

Table 3. 4 – Results of activity assays in *M. tuberculosis* H_{37}Rv performed by PhD Nuno Carmo from Host-Pathogen Interactions group of iMed ULisboa, lead by Professor Elsa Anes.

Compound	R	MIC_{90} (% of inhibition)/µM	%Inhibiton (100µM)
1	H	>100	9
2	C_2H_5	>100	23
3	C_4H_9	>100	8
4	C_6H_{13}	>100	59
5	C_9H_{19}	>100	79
6	$C_{10}H_{21}$	>100	80
7	$C_{11}H_{23}$	>100	76
8	$C_{12}H_{25}$	100	100
9	$C_{14}H_{29}$	>100	60
10	C_9H_{19}	>100	82
Compound	**R**	**MIC_{90} (% of inhibition)/µM**	**%Inhibiton (100µM)**
1a	H	>100	89
3a	C_4H_9	>100	84
5a	C_9H_{19}	100	93
6a	$C_{10}H_{21}$	50	94
7a	$C_{11}H_{23}$	100	90
8a	$C_{12}H_{25}$	100	92
9a	$C_{14}H_{29}$	>100	0
10a	C_9H_{19}	100	99

The results obtained show that most of the MICs obtained do not have an exact value. In library 1, only compound **8** inhibit more than 90% in the bacterial growth. Although, in library 2, compounds **5a**, **6a**, **7a**, **8a** and **10a** inhibit more than 90% of the bacterial growth.

The percentage of inhibition (**table 3.4**) was also calculated because MIC values give the minimum concentration to inhibit at least 90% of bacterial growth. Since some concentrations are higher than

100µM, it was interesting to understand if these compounds had some inhibition or if there was no activity at all. Through these values, it is possible to observe that compound **9a** does not inhibit the bacterial growth, within the used concentration range, so is inactive against the virulent strain H_{37}Rv of *M. tuberculosis* until the concentration of 100µM. Compounds **2** and **3** are active but are not strong inhibitors because the bacterial growth was just inhibited by 23% and 8%, respectively. All the other compounds inhibit more than 50% of the bacterial growth. Compounds **6**, **3a**, **5a**, **6a**, **7a**, **8a** and **10a** inhibit more than 80% so they have good activities against *M. tuberculosis* H_{37}Rv strain.

The MICs obtained showed that most of the compounds do not inhibit 90% of the bacterial growth with concentration of 100µM, so an estimation of 50% of inhibition was done, in order to observe which is the concentration that inhibits more than 50% of the bacterial growth. Compounds **1**, **2**, **3** and **9a**, with the maximum concentration used, show a low percentage of inhibition (below 50%) so it was not possible to estimate the concentration that inhibits more than 50% of the bacterial growth. The results of this estimation are shown in **figures 3.5** and **3.6**.

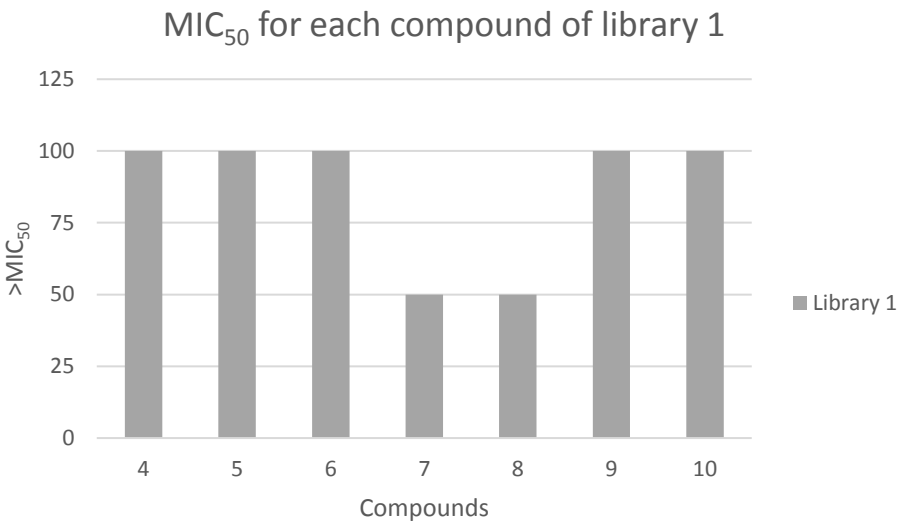


Figure 3. 5 - Estimation of 50% of inhibition of *M. tuberculosis* H_{37}Rv strain growth by compounds of library 1.

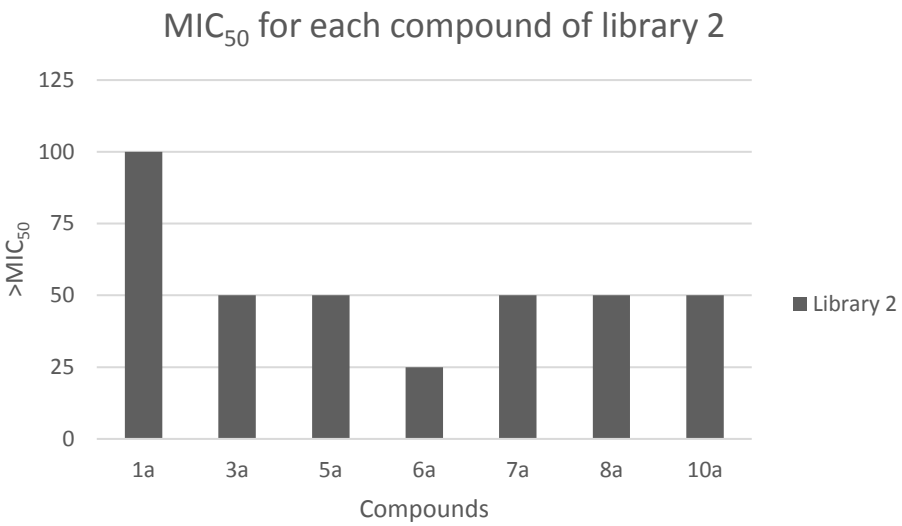


Figure 3. 6 - Estimation of 50% of inhibition of *M. tuberculosis* H_{37}Rv strain growth by compounds of library 1.

The results of 50% of inhition estimation for compounds of library 1 (**figure 3.5**) show that the best compounds are **7** and **8**, in this library. These compounds are needed in a concentration of 50µM to inhibit at least 50% of the bacterial growth. The other compounds are needed in a concentration of 100µM, so they are less actives.

In **figure 3.6**, there are presented the results of at least 50% of inhibition of bacterial growth for compounds of library 2. With these results, it is possible to conclude that compounds 6a is the most active because it inhibits at least 50% of bacterial growth with a concentration of 25µM. The other compounds are needed in a concentration of 50µM, except compound **1a** that is needed in 100µM.

These activity results *in vitro* show that compounds of library 2 have more activity against *M. tuberculosis* H_{37}Rv strain than compounds of library 1, because the MICs obtained are lower (and it was possible to measure most of them) and for those whose inhibitory concentration stays above the concentration range of the assays the inhibition percentages observed at the concentration maximum used are higher, even above 80% of inhibition.

Comparing both of the acids, compounds **1** and **1a**, it is possible to observe that the MICs obtained do not have an exact value so an estimation of >50% of inhibition was done. It is possible to conclude that acid **1a** is more active against *M. tuberculosis* H_{37}Rv strain than acid **1**. The inhibition resultant of the action of these acids in the bacteria are different because compound **1** only inhibits 9% of the bacterial growth and compound **1a** inhibits 89%, which reveals that this compound is much higher active than acid **1**. These results are concordant with the ones obtained for the esters (compounds of library 2 have more activity than compounds of library 1).

So it is possible to conclude that the substitution of the methyl group to a trichloromethyl group on carbon C_7 leads to more active compounds against *M. tuberculosis* H_{37}Rv strain.

Discussion

Discussion

4.1 Synthesis and Structure Analysis

In the present work, 16 different esters were synthetized and all of them are derivatives from NAL. To perform these synthesis, two different methods were used: Method A and Method B. Method A consists in the formation of the acyl chloride during 15 minutes and immediately the alcohol was added to obtain the desired ester; this last reaction is kept under reflux during 1h. In method B, first the acyl chloride was synthesized (in a 2h reaction kept under reflux) and after the alkoxyde of the correspondent alcohol was added to the acyl chloride to synthesize the ester. This last reaction was kept under reflux and controlled by thin layer chromatography (TLC) until the product (the ester) was formed.

The main difference between the esters of each library is the group in carbon C_7: in library 1 the methyl stays intact but it library 2 it was substituted by a trichloromethyl group (**Figure 4.1**). For library 1, 9 different esters were synthesized with different alkoxyl chains, from 2 until 14 carbons length. The yields were obtained within a range from 51% until 79%. For library 2, 6 different esters were synthesized with different alkoxyl chains, from 4 until 14 carbons length. The yields were obtained with a range from 5% to 35%.

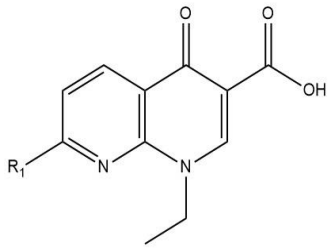
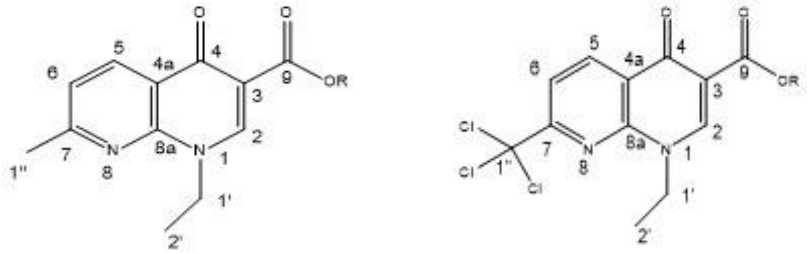


Figure 4. 1 - Starting material of the obtained compounds. In compound **1**, R_1 is a CH_3 group. In compound **1a**, R_1 is a CCl_3 group.

Characterization of all the compounds was done through NMR analysis. To identify the structures, ^{1}H, ^{13}C, APT, HMQC and HMBC were made to see all the signals and correlations. In **figure 4.2**, it is presented the general structure of compounds from both libraries with the main carbons numbered.



Library 1 Library 2

Figure 4. 2 - General structure of compounds from libraries 1 and 2 with the main carbons numbered.

After ^{1}H spectra analysis of the esters, it is possible to observe that the chemical shifts of the aromatic protons are different between the two libraries but they appeared in the same order: C_5-*H* > C_2-*H* > C_6-

H. Library 2 aromatic protons have a higher chemical shifts due to the substitution of a methyl group to a trichloromethyl in aromatic carbon C_7. This happens because chloro's electronegativity is much higher than hydrogen's, which means that a trichloromethyl group has a higher electronegativity than a methyl group[88]. The chemical shift of a NMR signal depends on the local magnetic environment of the nucleous and this is influenced by the electron density[53]. So, it was expected that the substitution of the methyl group for the trichloro group led to higher chemical shifts of the protons signal.

Acids (compounds **1** and **1a**) ^{1}-H spectra revealed different results in the chemical shifts of aromatic protons. In these compounds, the chemical shifts appeared as: C_2-*H* > C_5-*H* > C_6-*H*. These results showed that there is an exchange of position of C_5-*H* and C_2-*H*, when comparing with the esters spectra; that fact be due to the carboxylic acids be more polar than the esters so it will lead to a higher chemical shift for C_2-*H*, which is the aromatic proton near these organic functions.

The main difference between the ^{1}H spectra of the two libraries is the existence of a singlet at 2.58-2.66 ppm in library 1 that do not exist in library 2. This singlet corresponds to the methyl group attached to carbon C_7. In library 2, the hydrogens are substituted by three chloro atoms so that peak does not exist on ^{1}H spectra of all the compounds in this library.

All the compounds synthesized have similar spectra on the alkoxyl chain except compounds **10** and **10a** because these compounds have a methyl group attached do carbon $C_{1'''}$ (first carbon of the alkoxyl chain). So, in these compounds, this carbon is chiral, that means an asymmetric atom which implies that the two protons of the next carbon ($C_{2'''}$) are non-equivalent called diastereotopic. So, two different signals appeare d in the ^{1}H-NMR spectra. Therefore, instead of $C_{2'''}$-*H* signal be a multiplet, in compounds **10** and **10a** there are two multiplets for these protons, one for each hidrogen.

Coupling constants give us the information of which protons are coupled because these values are the same for signals from coupling protons[53]. So, coupling constants were calculated for duplets, triplets and quadriplets. With the values obtained, it is possible to conclude that the coupled protons are:

- $C_{5'}$-*H* and $C_{6'}$-*H*, with $J_{C5\text{-}H\text{-}C6\text{-}H} \approx 8.1 - 8.4$ Hz;
- $C_{1'}$-*H* and $C_{2'}$-*H*, with $J_{C1'\text{-}H\text{-}C2'\text{-}H} = 8.2$ Hz.

The other coupling constants obtained were not mentioned above because the protons couple with more than one group of protons leading to a multiplet signal and it is not possible to calculate constants for these kinds of signals.

In ^{13}C-NMR spectra results, the attribution of the signals to the right carbon was made with the help of APT spectra and also HMQC and HMBC correlations. APT spectra gives positive or negative peaks as a way to encode information about the number and type of protons attached to a carbon (C_q, CH, CH_2 or CH_3)[89]. These spectra were very useful to distinguish signals with similar chemical shifts: C_2 has a chemical shift of 149.78 ppm and C_{8a} has a chemical shift of 147.48 ppm so it only possible to made the right atribution with APT spectra. HMQC spectra correlates the protons in a molecule with the carbon atoms to which they are directly attached[90]. HMBC spectra correlates protons with nearby carbon atoms, not the carbon to which the proton is directly attached[90]. All these spectra (^{1}H, ^{13}C, APT, HMQC and HMBC) allowed understanding better the real structure of the compounds and to make the right attribution of all the carbons signals.

After the analysis of all the spectra, it was possible to observe that aromatic and C_9 carbons have a chemical shift between 110 and 178 ppm, that are the normal resonances for aromatic carbons and carbons of carboxylic acids attached to aromatic rings[91]. Besides that, it was also possible to observe that chemical shifts of $C_{1'}$ and $C_{2'}$ do not show relevant differences between both libraries of compounds

(46-48 ppm and 14-15 ppm, respectively). On the alkoxyl chain, the last carbon has a chemical shift of 13-14 ppm for all the esters, since the chemical shifts of CH_3 of alkane chains go down to low fields[91]. On the other hand, the carbon of the alkoxyl chain with higher chemical shift is the first, $C_{1'''}$, since is the one linked to the esters' oxygen. This carbon has a chemical shift between 60-71 ppm. In compounds **10** and **10a**, chemical shift of $C_{1'''}$ has the value of 71 ppm because this carbon becomes tertiary due to the methyl substitution instead of secondary as with the other esters.

Analysis of ^{13}C spectra only reveals one big difference between compounds of both libraries. In library 1, carbon $C_{1''}$ has a chemical shift of 24-25 ppm but in library 2 of 96 ppm. This difference happens because in library 1 this carbon is linked to three hydrogens and in library 2 is linked to three chloro atoms. Chloro atoms are more electronegative than hydrogen atoms so the chemical shift of the carbon which they are attached to increases[53,88,91].

Mass spectrometry analysis confirms the presence of the trichloromethyl group on carbon C_7 of compounds of library 2.

4.2 Stability Assays

Plasma and buffer stability assays were performed in order to evaluate enzymatic and chemical hydrolysis, respectively.

Primarily, all the compounds were diluted in ACN at different concentrations to do calibration curves in order to obtain the relation to convert the HPLC area values into concentrations on the other assays. After this, the esters were tested in human plasma and phosphate buffer suspensions. Samples were taken at different times and injected in the HPLC to observe the ester hydrolysis and the acid formation (**Figure 4.3**).

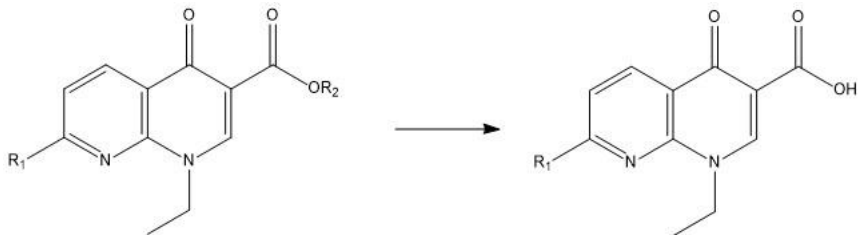


Figure 4. 3 – Ester hydrolysis in human plasma and phosphate buffer assays.

To calculate k_{obs} and $t_{1/2}$, a *pseudo*-first order kinetics was assumed because the kinetic decay observed was similar to a first order kinetics.

Human plasma assays were performed with 80% of human plasma suspension in phosphate buffer. These assays were done to evaluate compound's enzymatic stability. The results obtained show that all the esters are very stable in human plasma because some of them did not show any visible degradation (compounds **3**, **4**, **6**, **8**, **9**, **10**, **7a**, **9a** and **10a**) within the 72h of the assays and the rest of them have $t_{1/2}$ in the order of days. The less stable ester was **5a** with a $t_{1/2}$ of 2.8 days, which is yet a high $t_{1/2}$. When comparing the two libraries of compounds, it is possible to observe that compounds of library 1 are more stable, in general, than compounds in library 2. Since the only difference between them is the group in carbon C_7, it is possible to conclude that this difference is due to the introduction of a trichloromethyl group instead of a methyl group.

In phosphate buffer assays, the aim was to evaluate chemical hydrolysis, as it was mentioned above. The assays were performed with samples removed at different times. These results show that all the synthesized esters are very stable to chemical hydrolysis because all the $t_{1/2}$ obtained were in the order of days and the k_{obs} vary between 0.31 and 3.6 x10^3 h^{-1}. The most stable compound in these assays is compound **2**, with a k_{obs} of 0.31x10^3 h^{-1} and a $t_{1/2}$ of 92.7 days. On the other hand, the less stable compounds are **9** and **5a** with a k_{obs} of 3.6x10^3 h^{-1} and a $t_{1/2}$ of 7.8 days. Even among the compounds under study the less stable, these compounds are still very stable because their $t_{1/2}$ is quite high.

To evaluate the contribution of chemical hydrolysis in human plasma enzymatic hydrolysis, k_{obs} of the both assays were compared, calculating the ratio between k_{plasma}/k_{buffer}. This is very important because human plasma assays were performed in human plasma with phosphate buffer so it is necessary to estimate which is the contribution of phosphate buffer hydrolysis. For some esters it was not possible to calculate the ratios, since they are very stable in human plasma assays, they did not show visible degradation within the assays time so it was not possible to calculate k_{plasma} for them (compounds **3**, **4**, **6**, **8**, **9**, **10**, **7a**, **9a** and **10a**). Calculation of the ratios of compounds **2**, **5**, **7**, **3a**, **5a**, **6a** and **8a** (**table 8**, chapter 3.3) show that all of them have a value above 1. Compounds **2**, **7**, **5a** and **8a** have a higher enzymatic hydrolysis than chemical hydrolysis because obtained ratios vary between 2.8 and 7.5. For compounds **5**, **3a**, **5a** and **6a**, the ratios obtained are approximately 1, so it is possible to conclude that human plasma enzymatic hydrolysis is very similar to phosphate buffer chemical hydrolysis.

With these stability results it is possible to conclude that all the compounds under study are very stable. These results are very good since it is expected to these compounds to act on macrophages infected with *M. tuberculosis*, they must survive to plasma transportation in order to reach the target site[77].

4.3 Activity Assays

All the compounds have good results in stability assays so they were tested in order to evaluate their activity against *M. tuberculosis* H_{37}Rv strain.

H_{37} was originally isolated in 1905 and earned attention for its noted virulence in the guinea pig model. In 1934, H_{37} was dissociated into "virulent" (Rv) and "avirulent" (Ra) strains[92]. H_{37}Rv strain was used to evaluate the activity of the synthesized esters. To evaluate the activity of the compounds, it was measured the MIC_{90} in µM, which represents the minimum concentration the inhibits at least 90% of the bacterial growth. The results of activity studies are presented in **table 3.4**.

All the esters and the acids have activity against *M. tuberculosis* H_{37}Rv, except compound **9a**. The most active compound is compound **6a**, an ester with 10 carbons on the alkoxyl chain and a trichloromethyl substitute on carbon C_7. This compound has a MIC_{90} of 50µM.

Since most of the compounds do not show an exact concentration of MIC_{90}, it was analysed the inhibition of at least 50% of the bacterial growth. Compounds **1**, **2**, **3** and **9a** still do not have an exact value on this estimation, which means that they only inhibit less than 50% of the bacterial growth. The other compounds have activity at this inhibition percentage. The concentration needed to achieve this value of growth are low and the best result corresponds again to compound **6a**, that is only needed 25µM of it to inhibit at least 50% of the bacterial growth.

To evaluate better the activity studies results, the percentage of inhibition was also calculated for a concentration of 100µM (**table 4.1**).

Table 4. 1 – Percentage of inhibition of the bacterial growth *M. tuberculosis* H_{37}Rv strain to a concentration of 100µM.

Compound	%Inhibition
1	9
2	23
3	8
4	59
5	79
6	80
7	76
8	100
9	60
10	82
Compound	**%Inhibition**
1a	89
3a	84
5a	93
6a	94
7a	90
8a	92
9a	0
10a	99

With these percentages values, it is possible to observe that the compound with higher inhibition of the bacterial growth with a concentration of 100µM is compound **8**. This compound promotes an inhibition of 100%. The less active compounds in a concentration of 100µM are **1**, **2** and **3** with an inhibition of 9, 23 and 8%, respectively. Compound **9a** does not inhibit bacterial growth at the considered concentration. So, these four compounds are considered the worse in terms of inhibition of the growth of *M. tuberculosis* H_{37}Rv strain. Both libraries of compounds have good inhibition results but library 2 has higher inhibition percentages (between 84-99%, except compound **9a**) than library 1 (between 59-100%, except compounds **1**, **2** and **3**).

With these activity assays results, it is possible to conclude that compounds of library 2 are, in general, more active than compounds of library 1. The only difference between the two libraries of compounds is the trichloromethyl group instead of a methyl group. So, the difference of activities between the two libraries of compounds must be due to this substitution in carbon C_7.

All the synthesized compounds are NAL derivatives, so it is expected that they have the same target. NAL is a quinolone and the target of this kind of compounds in bacteria is DNA gyrase, a type II topoisomerase[93]. It is thought that the toxicity of quinolones in bacterial cell is due to the formation of a topoisomerase-drug-DNA ternary complex that cellular processes convert into a lethal lesion[94]. In fact, it is suggested that quinolones bind to multiple points along the helix-4 of DNA gyrase with carbon C_7 near position 81 (Figure 4.).In this position, the aminoacid is a glycine[95]. This aminoacid has a nonpolar side chain, that is an hydrogen[56]. It is also suggested that carboxyl and keto oxygens of quinolones are near GyrA, near Asp87 and Ser83 residues, respectively (**Figure 4.4**)[95].

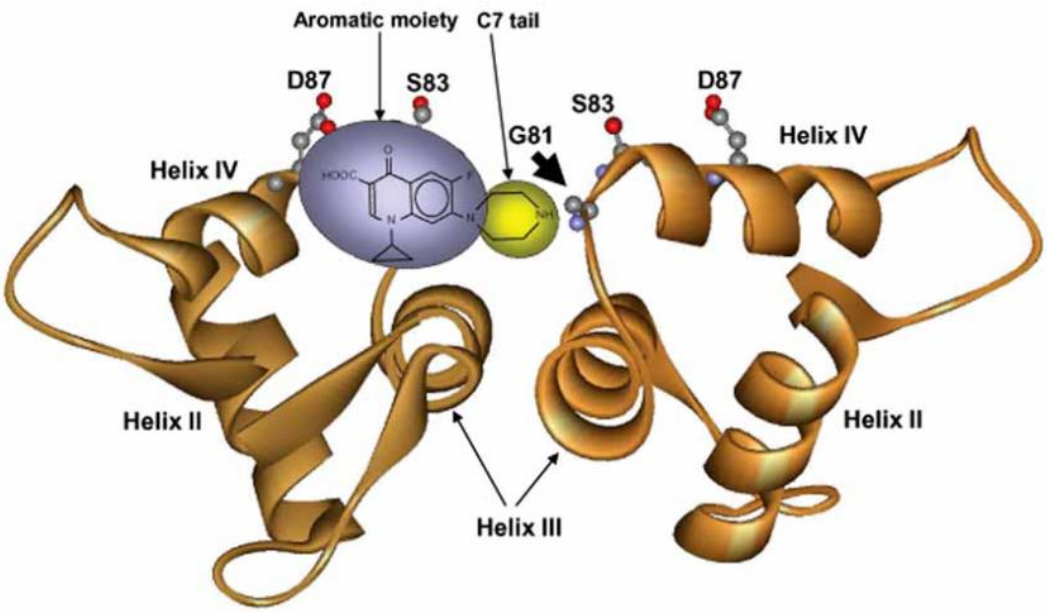


Figure 4. 4 - Model of quinolone binding to GyrA-GyrA dimer of DNA gyrase. Substitute on carbon C7 is near Gly81. Carboxyl and keto oxygens are near Asp87 and Ser83, respectively[95].

Although, there are several suggested mechanisms of DNA gyrase inhibition by quinolones so more studies are necessary to understand how these compounds are actives against *M. tuberculosis*.

The synthesized compounds in this study are derivatives of NAL. These compounds are esters so they are more lipophilic than NAL, which means that they may cross biological membranes easily[96]. It is expected that, after they cross the cell membrane of *M. tuberculosis*, they will be degradated by mycobacterial esterases. The product of this enzymatic hydrolysis is NAL, compound **1** or the synthesized derivative of it, compound **1a** for compounds of libraries 1 and 2, respectively.

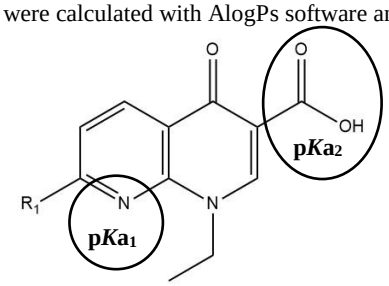
As it was mentioned before, they difference between compounds **1** and **1a** is the presence of a methyl group or a trichloromethyl group in carbon C_7, respectively. It is possible to observe that compounds of library 2 are more active than compounds of library 1 so this difference may be due to the trichloromethyl substitution.

4.4 Structure-activity relationship

There are some parameters that are very important in drug design. One of them is lipophilicity. Cell membranes are composed principally of phospholipid bilayers, that are formed with polar ionised head groups and a highly hydrophobic inner core. To cross this bilayer membrane, molecules must be hydrophobic[97]. So, lipophilicity represents the affinity of a molecule for a lipophilic environment. This characteristic is expressed by the partition coefficient (logP) that is defined as the ratio of concentrations of the compound at equilibrium between the organic and aqueous phases[97,98]. Esters are lipophilic molecules so they are good options as prodrugs to enhance the entrance of drugs inside the cell[54].

In this chapter, to sum all the data, it is established the relation between structural characteristics of all the synthesized compounds with their plasma stability and activity against *M. tuberculosis*. In **table 4.2** it is presented all the structural characteristics and also stability and activity results for each compound.

Table 4. 2 – Structural characteristics, plasma stability and activity against H_{37}Rv strain of *M. tuberculosis* results for each compound. The logP values were calculated with AlogPs software and p*K*a values with ACD/I-Lab. nd – no degradation.



Compound	R	logP	p*K*a$_1$	p*K*a$_2$	k_{plasma}	%degradation	%inhibition
1	H	0.95	4.5	6.2	-	-	9
2	C_2H_5	2.27			1.2	6	23
3	C_4H_9	3.13			nd	nd	8
4	C_6H_{13}	3.95			nd	nd	59
5	C_9H_{19}	5.28			1.2	6	79
6	$C_{10}H_{21}$	5.77	4.6	-	nd	nd	80
7	$C_{11}H_{23}$	6.34			2.9	15	76
8	$C_{12}H_{25}$	6.50			nd	nd	100
9	$C_{14}H_{29}$	6.84			nd	nd	60
10	$CH(CH_3)C_7H_{15}$	5.34			nd	nd	82
Compound	**R**	**logP**	**p*K*a$_1$**	**p*K*a$_2$**	**k_{plasma}**	**%degradation**	**%inhibition**
1a	H	2.78	4.5	6.1	-	-	89
3a	C_4H_9	4.5			3.1	16	84
5a	C_9H_{19}	6.39			10.2	53	93
6a	$C_{10}H_{21}$	6.75			1.2	6.2	94
7a	$C_{11}H_{23}$	7.07	4.6	-	nd	nd	90
8a	$C_{12}H_{25}$	7.30			8.3	13	92
9a	$C_{14}H_{29}$	7.80			nd	nd	0
10a	$CH(CH_3)C_7H_{15}$	6.35			nd	nd	99

Through **table 4.2**, it is possible to observe that the acids have two p*K*a values (p*K*a$_1$ and p*K*a$_2$) but the synthesized esters only have one (p*K*a$_1$). It was expected that the substitution on carbon C7 lead to a higher pKa_1 value but no differences were observed on the estimations. This *pKa* value is 4.6 which means that at pH 4.6 that half of the molecules are protonated in this position. These estimated *pKa* values indicate that compounds **1** and **1a** are weak acids. In fact, it was previously demonstrated that *M. tuberculosis* is susceptible to weak acids and to acid pH. The differential disruption of membrane in *M. tuberculosis* caused by weak acids could result from the slow metabolism and, consequently, slow energy production in the slow growing of the bacteria and a defective efflux mechanism[78].

The alkoxyl chain length of the derivatives may have influence in the activity of the compound against *M. tuberculosis* H_{37}Rv strain and in the stability in plasma.

Relationship between k_{obs} values and number of carbons of alkoxyl chain (**figure 4.5**), between inhibition percentage and number of carbons of alkoxyl chain (**figures 4.6** and **4.7**) and between inhibition percentage and lipophilicity (**figures 4.8** and **4.9**) are established bellow.

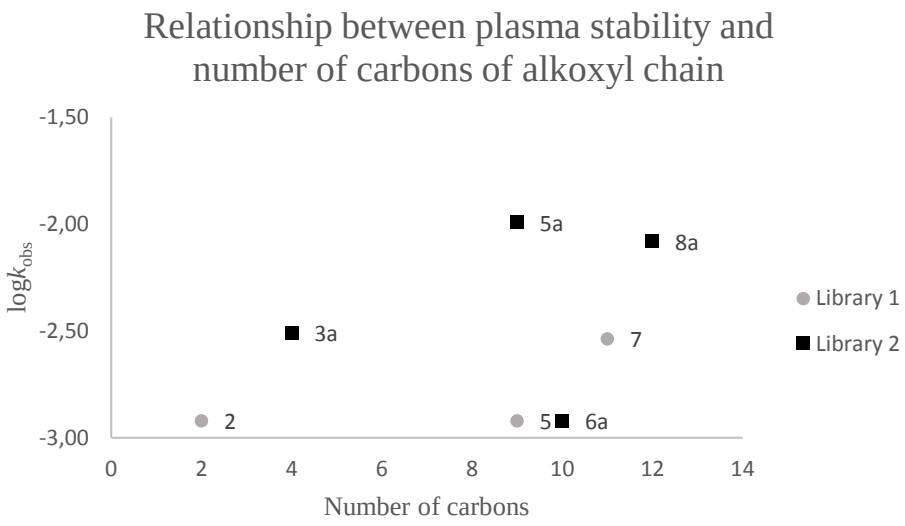


Figure 4. 5 – Relationship between alkoxyl chain length and k_{obs} value (plasma).

In **figure 4.5**, it is possible to observe that there is not a correlation between the length of alkoxyl chain and plasma stability. In library 1, there is no possible conclusion but in library 2, in general, it is possible to assume that, except for compound **6a**, when the alkoxyl chain is longer, plasma stability decreases. These results indicate that higher alkoxyl chains are not beneficial to stability.

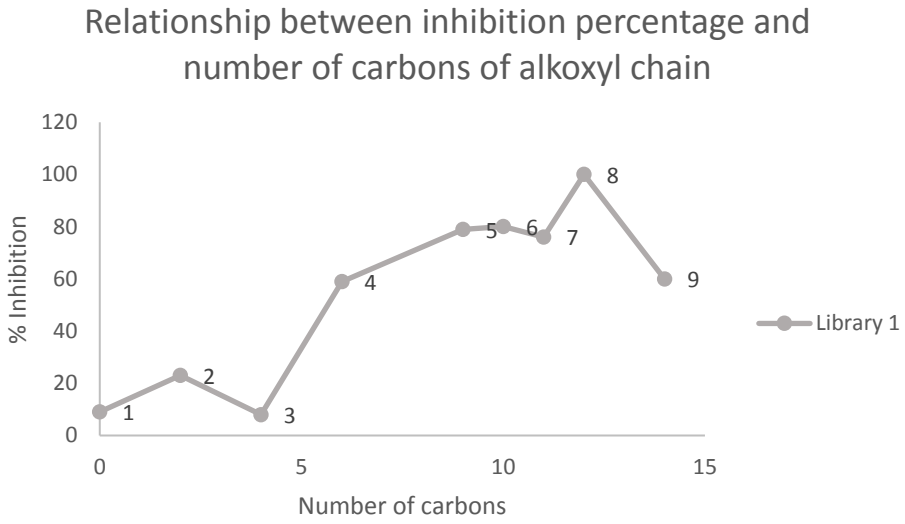


Figure 4. 6 - Relationship between alkoxyl chain length and inhibition percentage of *M. tuberculosis* H_{37}Rv growth for a concentration of 100µM of compounds of library 1.

The relationship between the inhibition percentage and number of carbons of the alkoxyl chain for compounds of library 1 is shown above (**figure 4.6**). In this library, it is possible to observe that when number of carbons of the alkoxyl chain is higher, the inhibition percentage is also higher, except for compound **9**. This result indicates that for this library of compounds higher alkoxyl chains lead to an increase on the activity of the compounds.

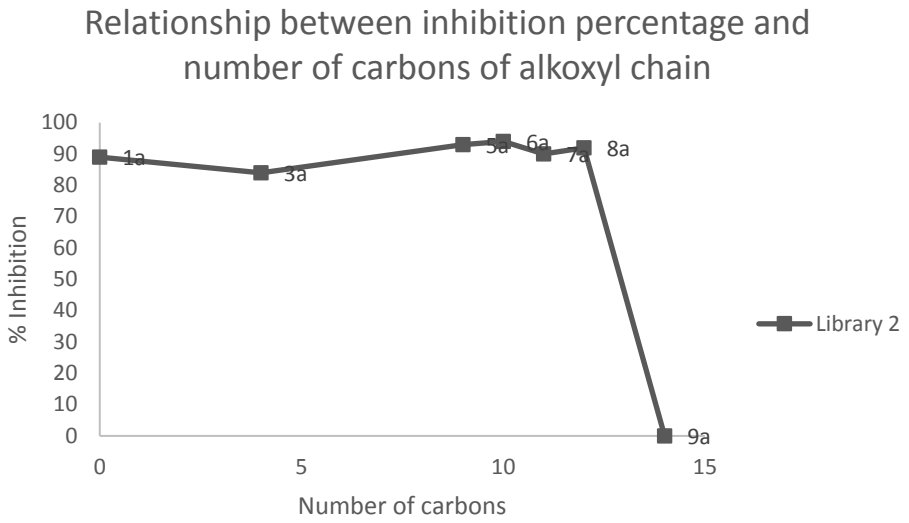


Figure 4. 7 - Relationship between alkoxyl chain length and inhibition percentage of *M. tuberculosis* H_{37}Rv growth for a concentration of 100µM of compounds of library 2.

For compounds of library 2 (**figure 4.7**) it is possible to conclude that the length of the alkoxyl chain have no direct influence on the activity results, except for compound **9a**. Since this compound has the biggest alkoxyl chain maybe it has no activity because it is very liposoluble. As it is possible to observe on the graphic above, the inhibition percentage of compounds from library 2 is similar between all of them so it is possible to conclude that the activity of these compounds is not mainly due to the length of the alkoxyl chain but to the fact that these compounds have a trichloromethyl group on carbon C_7. Since there is no relationship between inhibition percentage and alkoxyl chain length maybe there are some other factors that are important for transportation through membranes for these compounds, such as, membrane transporters.

Hydrophilic/Hydrophobic properties of a drug are crucial in influencing its solubility and ADMET[96]. Molecules that are very polar or too hydrophilic do not cross the cell membranes, that are constituted by phospholipids[96,97]. However, very hydrophobic drugs are likely to be dissolved in fat globules and are poorly absorbed, when they are administrated orally. On the other hand, if they are injected, they are poorly soluble in blood and are likely to be taken up by fat tissue, so the circulating levels of the drug are low[96]. So, it is necessary to find an equilibrium between hydrophilia and lipophilia of the drug.

As it was mentioned before, the property used to evaluate this characteristic of the drug is logP. Hydrophobic compounds have high logP values and hydrophilic have low values. In general, the susceptibility of *M. tuberculosis* to weak acids increases with the logP value of the compound, that is due to the fact that lipophilic prodrugs enter into the mycobacteria easily[99].

In **table 4.2** is possible to conclude that, as it is expected, the logP value increases with the size of the alkoxyl chain. This is due to the fact that esters with larger alkoxyl chains are more lipophilic so logP values increase. High values of logP are good because the synthesized compounds have to be lipophilic to cross the bacteria membrane that is constituted by mycolic acids that measure the sensitivity to hydrophobic antibiotics[13,54].

It is expected that compounds with higher logP values are more active against *M. tuberculosis* H_{37}Rv strain and, also, to have a higher stability in human plasma.

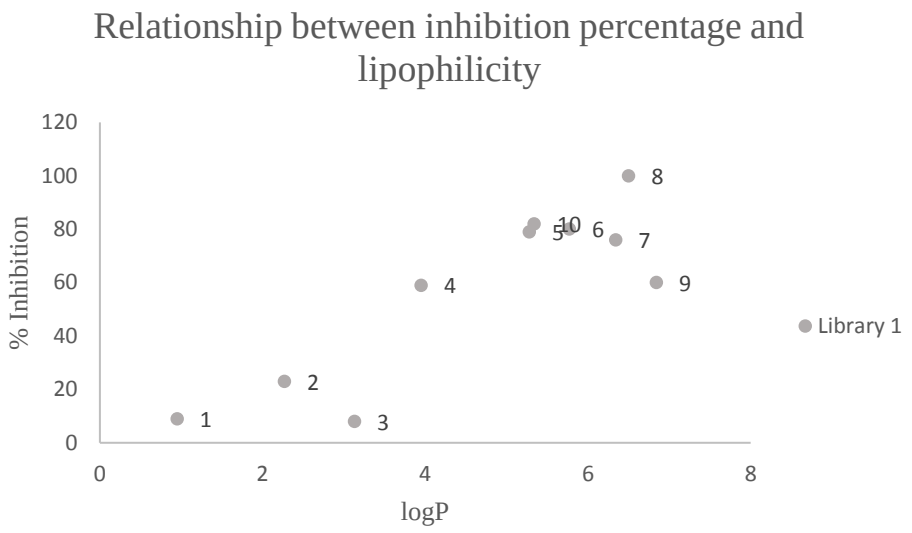


Figure 4. 8 - Relationship between alkoxyl logP and percentage of inhibition of *M. tuberculosis* H_{37}Rv strain growth for a concentration of 100µM for compounds of library 1.

In **figure 4.8**, it is possible to observe that, in general, when the logP value is higher, the inhibition percentage is also higher. The exceptions to this conclusion are compounds **3** (alkoxyl chain with 4 carbons length), **7** (alkoxyl chain with 11 carbons length) and **9** (alkoxyl chain with 14 carbons length).

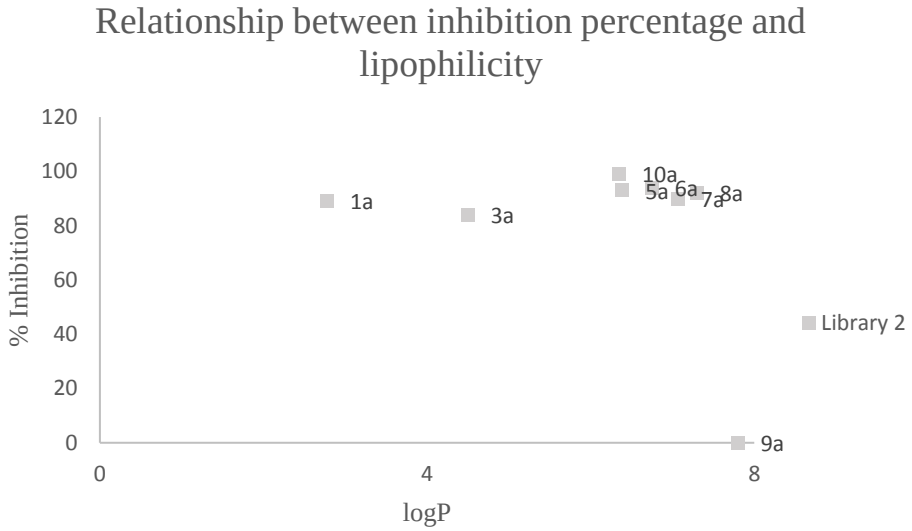


Figure 4. 9 - Relationship between logP and percentage of inhibition of *M. tuberculosis* H_{37}Rv strain growth for a concentration of 100µM for compounds of library 2.

In **figure 4.9**, it is shown the relation between inhibition growth of *M. tuberculosis* H_{37}Rv strain and lipophilia for compounds of library 2. By this figure, it is possible to observe that logP values do not have influence in the percentage inhibition, except for compound **9a**. As it is possible to observe, the inhibition percentage is almost the same for all the compounds, even for compounds with higher logP. These results, combined with the results of **figure 4.5** (for compounds of library 2) show that the activity

of these compounds may not be due to the lipophilia but to the existence of a trichloromethyl group in carbon C_7.

Compounds of library 2 are more lipophilic than compounds of library 1. In fact, compound **1a** is an acid and inhibits 89% of the bacterial growth. This compound has a logP value of 2.78 so it has a favourable logP to cross *M. tuberculosis* cell wall, which explains the difference of activity between this compound and NAL (compound **1**) that has a logP value of 0.95.

This conclusion arises because compounds of library 1 show differences in activity with the variation of logP and this does not happen with compounds of library 2. As it was mentioned before, the only difference between the two libraries is the substitution of a methyl group (in library 1) to a trichloromethyl group (in library 2) in carbon C_7. So, activity of compounds of library 2 can only be explained by the existence of this group.

In **figure 4.10** it is shown the relationship between plasma stability and lipophilicity.

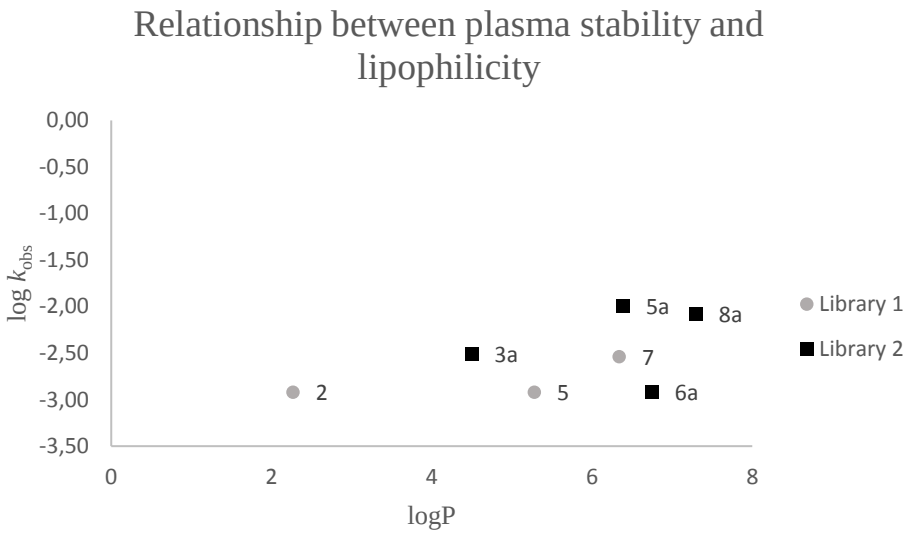


Figure 4. 10 – Relationship between lipophilicity and plasma stability.

Like the relationship between plasma stability and number of carbons of the alkoxyl chain, there is no relationship between plasma stability and lipophilicity for compounds of library 1. For compounds of library 2, it is possible to observe that more lipophilic compounds are less stable in plasma, except for compound **6a**. Although, there was not possible to obtain more k_{obs} because most of the compounds did not show degradation in human plasma assays within the 72h of assay. So, since there are only a few values of k_{obs}, there was not possible to conclude something in particular about the relationship between plasma stability and lipophilicity/number of carbons of the alkoxyl chain.

Conclusion

4. Conclusion

In the synthesis of NAL derivatives two methods were performed: method A and method B. Method A consists in the addiction of NAL to thionyl chloride and addiction of the alcohol 15 minutes later. In method B, NAL reacts with thionyl chloride for 2h and the alkoxide ion, previously synthesized, is added. Method A was used to synthesize compounds of library 1, compounds with an alkoxyl chain between 2 and 14 carbons length. The yields obtained were in a range between 51% (compound **5**) and 79% (compound **10**). Compounds of library 2 have an alkoxyl chain between 4 and 14 carbons length and were synthesized with method B. The yields obtained for this library were in a range between 5% (compounds **5a** and **6a**) and 35% (compound **9a**). So it is possible to conclude that, with method A, the yields of compounds are much better than the yields of compounds synthesized with method B.

One of the most important aims of prodrugs is their stability in human plasma because it is necessary that they achieve the pretended target without being degradated by human enzymes. In this work, all the compounds are very stable in human plasma being the less stable compound is **5a** with a $t_{1/2}$ of 2.8 days and 53% of degradation in 72h. Compounds **3**, **4**, **6**, **8**, **9**, **10**, **7a**, **9a** and **10a** are the most stable because they did not show any degradation within the 72h of assay.

Comparison of *pseudo*-first order constants obtained in human plasma and phosphate buffer assays give the contribution of chemical hydrolysis in enzimatic hydrolysis. It is possible to conclude that, for compounds **2**, **7**, **5a** and **8a**, the contribution of chemical hydrolysis is low because the ratios obtained were above 2. For compounds **5**, **3a** and **6a**, the ratios obtained were similar to 1 so it is possible to conclude that chemical hydrolysis has the same contribution on as the enzymatic hydrolysis.

It is important that compounds have a good plasma stability but they have to be active against *M. tuberculosis*. Studies in *M. tuberculosis* H_{37}Rv strain showed that all the compounds, except compound **9a**, are active against the bacteria. This means that the compounds can enter the bacterial cell envelope and might be activated (transformation of the ester in the correspondent carboxylic acid) inside it.

Compounds **8**, **5a**, **6a**, **7a**, **8a** and **10a** were the ones with best results in activity assays. All of them have a MIC_{90} of 100µM, except compound **5a** that has a MIC_{90} of 50µM. So, compound **5a** is the most active. When analyzing the percentages of inhibition of bacterial growth at a concentration of 100µM, it is possible to observe that the percentages of inhibition are quite high. In fact, the compounds that show lower inhibition on the bacterial growth are compounds **1** (9%), **2** (23%), **3** (8%) and **9a** (0%). All the other compounds inhibit more than 59% of the bacterial growth. One interesting result is that compound **1a**, that is not an ester, but inhibits 89%. With these results, it is possible to conclude that compounds of library 2 are more active against H_{37}Rv *M. tuberculosis* strain than compounds of library 1.

The most promising compound is **6a**. This compound had a $t_{1/2}$ of 24.2 days in human plasma assays, so it is very stable to enzymatic hydrolysis and it was thr most active compound tested against *M. tuberculosis* H_{37}Rv strain.

To conclude, the main objective of the present work was achieved because NAL derivatives were synthesized and the compounds are quite resistant to plasmatic proteins hydrolysis and are active against *M. tuberculosis*.

Future Perspectives

5. Future perspectives

In the future, more studies with these compounds are necessary. Firstly, studies in liver homogenate are necessary in order to confirm stability of compounds with hepatic enzymes. Studies in mycobacteria homogenate are also necessary to confirm that mycobacterial esterases are capable of activate the compounds. The activity assays *in vitro* against *M. tuberculosis* H_{37}Rv strain showed that most of the compounds can inhibit bacterial growth, which means that they might be activated but it is necessary to confirm this fact. Besides that, it is necessary to evaluate which compounds are easily activated by mycobacterial esterases.

Another type of studies that has to be performed is toxicity studies. It is necessary to evaluate if the synthesized compounds are toxic for humans, to understand if these compounds are suitable to administration or not. There are also necessary modelation studies to understand how the compounds, by themselves or the correspondent acid, interact with the DNA gyrase and/or with DNA molecules by some type of complex. Some studies refer that quinolones are sterilizing agents which means that they kill the bacteria definitely[100]. So, activity studies should include sterilazation analysis.

In the end, *in vivo* studies must be executed.

Experimental Procedure

6. Experimental Procedure

7.1 Equipment and material

NMR spectra were done on Magnet System 300 MHz/54 mm Ultrashield, long hold time Bruker. The chemical shifts are expressed in ppm (parts per million) and the coupling constants (J) in Hertz (Hz). The spectra obtained are presented in the followed form: Nucleus (solvent): chemical shift (δ, ppm)[multiplicity (s- singlet, d- doublet, t- triplet, q- quartet, m- multiplet], coupling constant (*J*, Hz), relative intensity (nH, for number of protons), molecule attribution]. To characterize the compounds, ^{1}H and ^{13}C spectra were performed.

The melting points were measured on Bock-Monoscop <M> apparatus.

The HPLC studies were performed on VWR chromatograph constituted with two pumps VWR-Hitachi L-2160U, one UV detector VWR-Hitachi L-2400U, one column oven VWR-Hitachi L-2300 and one autosampler VWR-Hitachi L-2200U. The column used was LiChroCART ^{R}250-4 HPLC Cartridge LiChrospher^{R} 100 reversed phase RP-8 (5 µm) MERCK. The analysis of the chromatograms was done with Agilent EZ Chrom Elite software.

To perform Thin Layer Chromatography (TLC), used to follow reactions and column chromatography, aluminium sheets with silica gel 60 F_{254} (Merck) were used. The revelation of the chromatograms was performed with UV light 366 nm and a camera that contains a mixture of silica and iodide.

Columns of glass (different sizes) filled with silica gel 60 (0.063-0.200 nm) were used in order to isolate and purify the compounds.

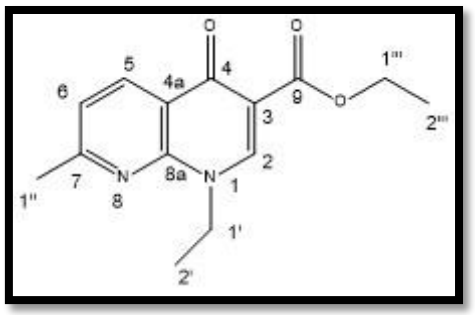
7.2 Reagents and Solvents

The solvents used were acquired from: José Manuel Gomes dos Santos Lda and António M. S. Cruz Lda.

The reagents used were acquired from: Sigma-Aldrich® and Fluka®.

7.3 Synthesis

Ethyl 1-ethyl-1,4-dihydro-7-methyl-1,8-naphthyridine-4-oxo-3-carboxylate (2)

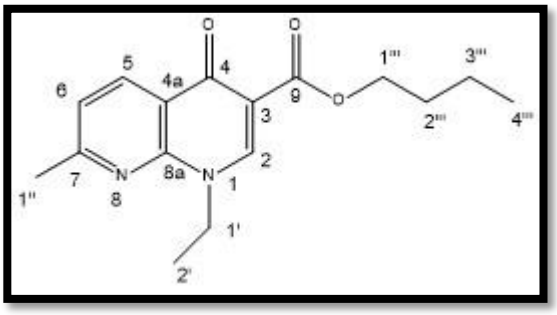


To synthesize this derivative, 82.70 mmol (6.00 mL) of thionyl chloride was added to 4.35 mmol (1.01 g) of NAL and the flask was kept aside for 15 minutes. Later 0.12 mol (6.80 mL) of ethanol was added to the solution, drop by drop and mixed thoroughly after each addition, and the reaction was kept for refluxing during 1h.

Then, the excess of thionyl chloride was evaporated and the organic phase was obtained after washing with 2x10 mL of sodium chlorine, 3x10mL of sodium bicarbonate and 3x5mL of ethyl acetate and drying with sodium sulfate anhydrous. Then, the solvent was evaporated and the organic phase was purified by column chromatography (9/1 to 1/1, hexane/ethyl acetate).

White solid, **η**= 65%
^{1}H-NMR δ (300 MHz, $CDCl_3$): 8.65 (*d*, *J*=8.1 Hz, 1H, C_5-*H*); 8.63 (*s*, 1H, C_2-*H*); 7.24 (*d*, *J*=8.1 Hz, 1H, C_6-*H*); 4.49 (*q*, *J*=7.2 Hz, 2H, $C_{1'}$-*H*); 4.41 (*t*, J=7.2 Hz, 2H, $C_{1'''}$-*H*); 2.66 (*s*, 3H, $C_{1''}$-*H*); 1.50 (*t*, *J*=7.2 Hz, 3H, $C_{2'}$-*H*); 1.42 (*t*, *J*=7.2 Hz, 3H, $C_{2'''}$-*H*)
^{13}C-NMR δ (75.48 MHz, $CDCl_3$): 174.74 (C_4); 165.64 (C_9); 162.66 (C_7); 148.74 (C_2); 148.58 (C_{8a}); 136.86 (C_5); 121.51 (C_{4a}); 121.14 (C_6); 112.48 (C_3); 60.81 ($C_{1'''}$); 46.53 ($C_{1'}$); 24.96 ($C_{1''}$); 15.03 ($C_{2'}$); 14.36 ($C_{2'''}$)
Melting point: 128-130ºC

Butyl 1-ethyl-1,4-dihydro-7-methyl-1,8-naphthyridine-4-oxo-3-carboxylate (3)



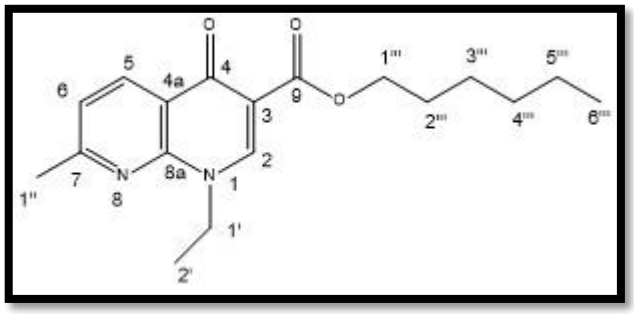
To synthesize this derivative, 82.70 mmol (6.00 mL) of thionyl chloride was added to 4.35 mmol (1.01 g) of NAL and the flask was kept aside for 15 minutes. Later 0.15 mol (10.98 g) of 1-butanol was added to the solution, drop by drop and mixed thoroughly after each addition, and the reaction was kept for refluxing during 1h.

Then, the excess of thionyl chloride was evaporated and the organic phase was obtained after washing with 2x10 mL of sodium chlorine, 3x10mL of sodium bicarbonate and 4x5mL of ethyl acetate and drying with sodium sulfate anhydrous. Then, the solvent was evaporated and the organic phase was purified by column chromatography (7/3 to 1/1, hexane/ethyl acetate).

White solid, η= 68%
^{1}H-NMR δ (300 MHz, $CDCl_3$): 8.63 (*d*, *J*= 8.1 Hz, 1H, C_5-*H*); 8.60 (*s*, 1H, C_2-*H*); 7.23 (*d*, *J*= 8.1 Hz, 1H, C_6-*H*); 4.48 (*q*, *J*= 7.2 Hz, 2H, $C_{1'}$-*H*); 4.33 (*t*, *J*= 6.6 Hz, 2H, $C_{1'''}$-*H*); 2.65 (*s*, 3H, $C_{1''}$-*H*); 1.77 (*m*, 2H, $C_{2'''}$-*H*); 1.48 (*m*, 5H, $C_{3'''}$-*H* and $C_{2'}$-*H*); 0.96 (*t*, *J*= 7.8 Hz, $C_{4'''}$-*H*)

^{13}C-NMR δ (75.48 MHz, $CDCl_3$): 174.71 (C_4); 165.77 (C_9); 162.62 (C_7); 148.68 (C_2); 148.60 (C_{8a}); 136.86 (C_5); 121.52 (C_{4a}); 121.10 (C_6); 112.06 (C_3); 64.84 ($C_{1'''}$); 46.57 ($C_{1'}$); 30.83 ($C_{2'''}$); 25.08 ($C_{1''}$); 19.29 ($C_{3'''}$); 15.22 ($C_{2'}$); 13.83 ($C_{4'''}$)
Melting point: 94-96ºC

Hexyl 1-ethyl-1,4-dihydro-7-methyl-1,8-naphthyridine-4-oxo-3-carboxylate (4)

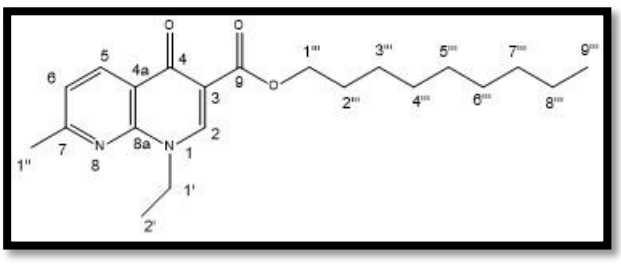


To synthesize this derivative, 82.70 mmol (6.00 mL) of thionyl chloride was added to 4.31 mmol (1.00 g) of NAL and the flask was kept aside for 15 minutes. Later 0.15 mol (15.16 g) of 1-hexanol was added to the solution, drop by drop and mixed thoroughly after each addition, and the reaction was kept for refluxing during 1h.

Then, the excess of thionyl chloride was evaporated and the organic phase was obtained after washing with 10 mL of sodium chlorine, 2x10mL of sodium bicarbonate and 3x5mL of ethyl acetate and drying with sodium sulfate anhydrous. Then, the solvent was evaporated and the organic phase was purified by column chromatography (7/3 to 1/1, hexane/ethyl acetate).

White solid, η= 59%
^{1}H-NMR δ (300 MHz, $CDCl_3$): 8.57 (*d*, *J*= 8.1 Hz, 1H, C_5-*H*); 8.54 (*s*, 1H, C_2-*H*); 7.16 (*d*, *J*= 8.1 Hz, 1H, C_6-*H*); 4.41 (*q*, *J*= 7.2 Hz, 2H, $C_{1'}$-*H*); 4.26 (*t*, *J*= 6.9 Hz, 2H, $C_{1'''}$-*H*); 2.59 (*s*, 3H, $C_{1''}$-*H*); 1.72 (*m*, 2H, $C_{2'''}$-*H*); 1.42 (*t*, *J*= 7.2 Hz, 3H, $C_{2'}$-*H*); 1.30-1.23 (*m*, 6H, $C_{3'''}$-*H*, $C_{4'''}$-*H* and $C_{5'''}$-*H*); 0.82 (*t*, *J*= 6.9 Hz, 3H, $C_{6'''}$-*H*)
^{13}C-NMR δ (75.48 MHz, $CDCl_3$): 174.74 (C_4); 165.72 (C_9); 162.63 (C_7); 148.66 (C_2); 148.62 (C_{8a}); 136.89 (C_5); 121.53 (C_{4a}); 121.11 (C_6); 112.06 (C_3); 65.16 ($C_{1'''}$); 46.57 ($C_{1'}$); 31.52 ($C_{2'''}$); 28.73 ($C_{3'''}$); 25.68 ($C_{4'''}$); 25.08 ($C_{1''}$); 22.55 ($C_{5'''}$); 15.21 ($C_{2'}$); 14.06 ($C_{6'''}$)
Melting point: 70-73ºC

Nonyl 1-ethyl-1,4-dihydro-7-methyl-1,8-naphthyridine-4-oxo-3-carboxylate (5)



To synthesize this derivative, 16.54 mmol (1.20 mL) of thionyl chloride was added to 0.86 mmol (0.20 g) of NAL and the flask was kept aside for 15 minutes. Later 29.67 mmol (4.28 g) of 1-nonanol was added to the solution, drop by drop and mixed thoroughly after each addition, and the reaction was kept for refluxing during 1h.

Then, the excess of thionyl chloride was evaporated and the organic phase was obtained after washing with 2x10 mL of sodium chlorine, 3x10mL of sodium bicarbonate and 2x5mL of ethyl acetate and drying with sodium sulfate anhydrous. Then, the solvent was evaporated and the organic phase was purified by column chromatography (7/3 to 1/1, hexane/ethyl acetate).

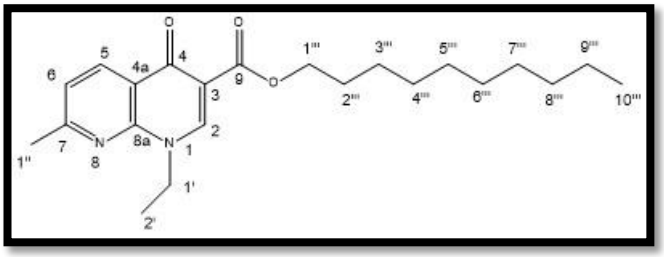
White solid, η= 51%

^{1}H-NMR δ (300 MHz, $CDCl_3$): 8.57 (*d*, *J*= 8.1 Hz, 1H, C_5-*H*); 8.53 (*s*, 1H, C_2-*H*); 7.16 (*d*, *J*= 8.1 Hz, 1H, C_6-*H*); 4.41 (*q*, *J*= 7.2 Hz, 2H, $C_{1'}$-*H*); 4.25 (*t*, *J*= 6.9 Hz, 2H, $C_{1'''}$-*H*); 2.58 (*s*, 3H, $C_{1''}$-*H*); 1.72 (m, 2H, $C_{2'''}$-*H*); 1.42 (t, *J*= 7.2 Hz, 3H, $C_{2'}$-*H*); 1.30-1.13 (m, 12H, $C_{3'''}$-*H*-$C_{8'''}$-*H*); 0.80 (t, *J*= 7.2 Hz, 3H, $C_{9'''}$-*H*)

^{13}C-NMR δ (75.48 MHz, $CDCl_3$): 174.73 (C_4); 165.72 (C_9); 162.62 (C_7); 148.66 (C_2); 148.61 (C_{8a}); 136.89 (C_5); 121.54 (C_{4a}); 121.10 (C_6); 112.07 (C_3); 65.17 ($C_{1'''}$); 46.57 ($C_{1'}$); 31.87 ($C_{2'''}$); 29.49 ($C_{3'''}$); 29.35 ($C_{4'''}$); 29.29 ($C_{5'''}$); 28.77 ($C_{6'''}$); 26.00 ($C_{7'''}$); 25.08 ($C_{1''}$); 22.67 ($C_{8'''}$); 15.21 ($C_{2'}$); 14.12 ($C_{9'''}$);
Melting point: 76-79ºC

Decyl 1-ethyl-1,4-dihydro-7-methyl-1,8-naphthyridine-4-oxo-3-carboxylate (6)

To synthesize this derivative, 16.54 mmol (1.20 mL) of thionyl chloride was added to 0.86 mmol (0.20 g) of NAL and the flask was kept aside for 15 minutes. Later 29.69 mmol (4.70 g) of 1-decanol was added to the solution, drop by drop and mixed thoroughly after each addition, and the reaction was kept for refluxing during 1h.



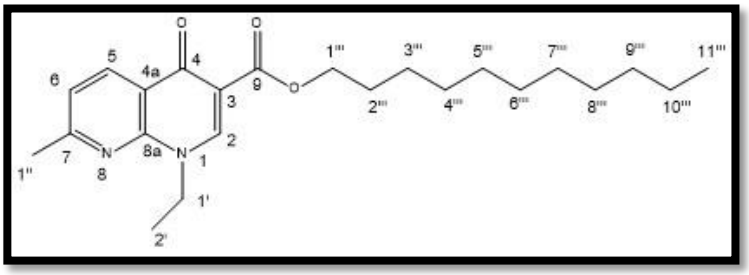
Then, the excess of thionyl chloride was evaporated and the organic phase was obtained after washing with 2x10 mL of sodium chlorine, 3x10mL of sodium bicarbonate and 5mL of dichloromethane and drying with sodium sulfate anhydrous. Then, the solvent was evaporated and the organic phase was purified by column chromatography (7/3 to 1/1, hexane/ethyl acetate).

White solid, η= 60%

^{1}H-NMR δ (300 MHz, $CDCl_3$): 8.65 (*d*, *J*= 8.1 Hz, 1H, C_5-*H*); 8.61 (*s*, 1H, C_2-*H*); 7.24 (*d*, *J*= 8.1 Hz, 1H, C_6-*H*); 4.48 (*q*, *J*= 7.2 Hz, 2H, $C_{1'}$-*H*); 4.33 (*t*, *J*= 6.9 Hz, 2H, $C_{1'''}$-*H*); 2.66 (*s*, 3H, $C_{1''}$-*H*); 1.79 (*m*, 2H, $C_{2'''}$-*H*); 1.49 (*t*, *J*= 7.2 Hz, 3H, $C_{2'}$-*H*); 1.36-1.24 (m, 14H, $C_{3'''}$-*H*-$C_{9'''}$-*H*); 0.87 (t, J= 6.9 Hz, 3H, $C_{10'''}$-*H*)

^{13}C-NMR δ (75.48 MHz, $CDCl_3$): 174.72 (C_4); 165.73 (C_9); 162.62 (C_7); 148.66 (C_2); 148.62 (C_{8a}); 136.89 (C_5); 121.54 (C_{4a}); 121.10 (C_6); 112.07 (C_3); 65.17 ($C_{1'''}$); 46.57 ($C_{1'}$); 31.89 ($C_{2'''}$); 29.58 ($C_{3'''}$); 29.53 ($C_{4'''}$); 29.34 ($C_{5'''}$); 29.32 ($C_{6'''}$); 28.77 ($C_{7'''}$); 25.99 ($C_{8'''}$); 25.08 ($C_{1''}$); 22.68 ($C_{9'''}$); 15.21 ($C_{2'}$); 14.12 ($C_{10'''}$)
Melting point: 85-89ºC

Undecyl 1-ethyl-1,4-dihydro-7-methyl-1,8-naphthyridine-4-oxo-3-carboxylate (7)

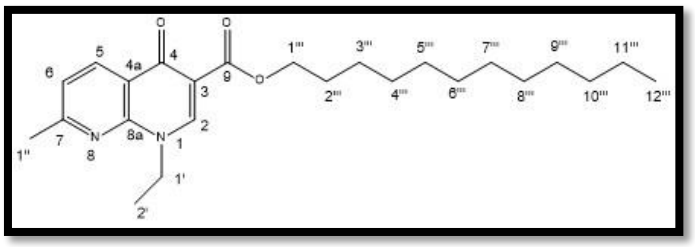


To synthesize this derivative, 16.54 mmol (1.20 mL) of thionyl chloride was added to 0.86 mmol (0.20 g) of NAL and the flask was kept aside for 15 minutes. Later 29.71 mmol (5.12 g) of 1-undecanol was added to the solution, drop by drop and mixed thoroughly after each addition, and the reaction was kept for refluxing during 1h.

Then, the excess of thionyl chloride was evaporated and the organic phase was obtained after washing with 2x10 mL of sodium chlorine, 3x10mL of sodium bicarbonate and 3x5mL of dichloromethane and drying with sodium sulfate anhydrous. Then, the solvent was evaporated and the organic phase was purified by column chromatography (7/3 to 1/1, hexane/ethyl acetate).

White solid, η= 65%
^{1}H-NMR δ (300 MHz, $CDCl_3$): 8.65 (*d*, *J*= 8.1 Hz, 1H, C_5-*H*); 8.61 (*s*, 1H, C_2-*H*); 7.24 (*d*, *J*= 8.1 Hz, 1H, C_6-*H*); 4.48 (*q*, *J*= 7.2 Hz, 2H, $C_{1'}$-*H*); 4.33 (*t*, *J*= 6.9 Hz, 2H, $C_{1'''}$-*H*); 2.66 (*s*, 3H, $C_{1''}$-*H*); 1.79 (*m*, 2H, $C_{2'''}$-*H*); 1.49 (*t*, *J*= 7.2 Hz, 3H, $C_{2'}$-*H*); 1.37-1.26 (m, 16H, $C_{3'''}$-*H*-$C_{10'''}$-*H*); 0.88 (t, *J*= 7.2 Hz, 3H, $C_{11'''}$-*H*)
^{13}C-NMR δ (75.48 MHz, $CDCl_3$): 174.71 (C_4); 165.74 (C_9); 162.61 (C_7); 148.66 (C_2); 148.62 (C_{8a}); 136.89 (C_5); 121.55 (C_{4a}); 121.09 (C_6); 112.09 (C_3); 65.18 ($C_{1'''}$); 46.56 ($C_{1'}$); 31.91 ($C_{2'''}$); 29.63 ($C_{3'''}$ and $C_{4'''}$); 29.53 ($C_{5'''}$); 29.35 ($C_{6'''}$ and $C_{7'''}$); 28.77 ($C_{8'''}$); 26.00 ($C_{9'''}$); 25.08 ($C_{1''}$); 22.69 ($C_{10'''}$); 15.22 ($C_{2'}$); 14.13 ($C_{11'''}$)
Melting point: 72-74ºC

Dodecyl 1-ethyl-1,4-dihydro-7-methyl-1,8-naphthyridine-4-oxo-3-carboxylate (8)



To synthesize this derivative, 16.54 mmol (1.20 mL) of thionyl chloride was added to 0.86 mmol (0.20 g) of NAL and the flask was kept aside for 15 minutes. Later 29.62 mmol (5.52 g) of 1-dodecanol was added to the solution, drop by drop and mixed thoroughly after each addition, and the reaction was kept for refluxing during 1h.

Then, the excess of thionyl chloride was evaporated and the organic phase was obtained after washing with 2x10 mL of sodium chlorine, 3x10mL of sodium bicarbonate and 5mL of dichloromethane and drying with sodium sulfate anhydrous. Then, the solvent was evaporated and the organic phase was purified by column chromatography (7/3 to 1/1, hexane/ethyl acetate).

Yellow solid, η= 66%
^{1}H-NMR δ (300 MHz, $CDCl_3$): 8.65 (*d*, *J*= 8.1 Hz, 1H, C_5-*H*); 8.61 (*s*, 1H, C_2-*H*); 7.24 (*d*, *J*= 8.1 Hz, 1H, C_6-*H*); 4.48 (*q*, *J*= 7.2 Hz, 2H, $C_{1'}$-*H*); 4.33 (*t*, *J*= 6.9 Hz, 2H, $C_{1'''}$-*H*); 2.66 (*s*, 3H, $C_{1''}$-*H*); 1.79

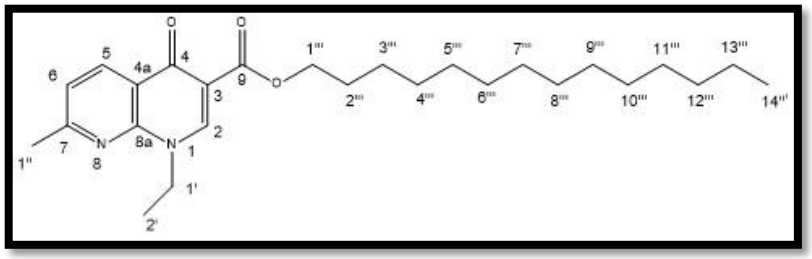
(*m*, 2H, $C_{2'''}$-*H*); 1.49 (*t*, *J*= 7.2 Hz, 3H, $C_{2'}$-*H*); 1.37-1.26 (*m*, 18H, $C_{3'''}$-*H*-$C_{11'''}$-*H*); 0.88 (*t*, *J*= 6.9 Hz, 3H, $C_{12'''}$-*H*)

^{13}C-NMR δ (75.48 MHz, $CDCl_3$): 174.73 (*C_4*); 165.72 (*C_9*); 162.63 (*C_7*); 148.66 (*C_2*); 148.61 (*C_{8a}*); 136.89 (*C_5*); 121.54 (*C_{4a}*); 121.11 (*C_6*); 112.07 (*C_3*); 65.18 (*$C_{1'''}$*); 46.57 (*$C_{1'}$*); 31.92 (*$C_{2'''}$*); 29.67 (*$C_{3'''}$*); 29.64 (*$C_{4'''}$* and *$C_{5'''}$*); 29.53 (*$C_{6'''}$*); 29.35 (*$C_{7'''}$* and *$C_{8'''}$*); 28.78 (*$C_{9'''}$*); 26.00 (*$C_{10'''}$*); 25.08 (*$C_{1''}$*); 22.69 (*$C_{11'''}$*); 15.22 (*$C_{2'}$*); 14.13 (*$C_{12'''}$*)

Melting point: 61-64ºC

Tetradecyl 1-ethyl-1,4-dihydro-7-methyl-1,8-naphthyridine-4-oxo-3-carboxylate carboxylate (9)

To synthesize this derivative, 16.54 mmol (1.20 mL) of thionyl chloride was added to 0.86 mmol (0.20 g) of NAL and the flask was kept aside for 15 minutes. Later 29.67 mmol (6.36 g) of 1-tetradecanol was added to the solution, drop by drop and mixed thoroughly after each addition, and the reaction was kept for refluxing during 1h.



Then, the excess of thionyl chloride was evaporated and the organic phase was obtained after washing with 2x10 mL of sodium chlorine, 3x10mL of sodium bicarbonate and 2x5mL of dichloromethane and drying with sodium sulfate anhydrous. Then, the solvent was evaporated and the organic phase was purified by column chromatography (7/3 to 1/1, hexane/ethyl acetate).

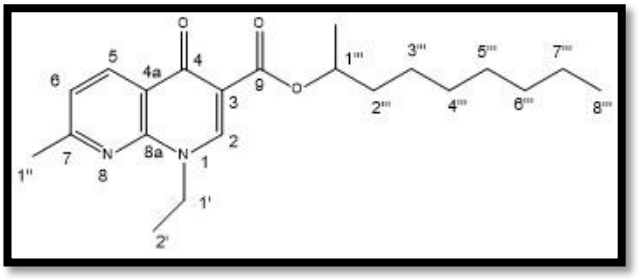
Yellow solid, η= 63%

^{1}H-NMR δ (300 MHz, $CDCl_3$): 8.57 (*d*, *J*= 8.1 Hz, 1H, C_5-*H*); 8.54 (*s*, 1H, C_2-*H*); 7.17 (*d*, *J*= 8.1 Hz, 1H, C_6-*H*); 4.41 (*q*, *J*= 7.2 Hz, 2H, $C_{1'}$-*H*); 4.25 (*t*, *J*= 6.9, 2H, $C_{1'''}$-*H*); 2.59 (*s*, 3H, $C_{1''}$-*H*); 1.72 (*m*, 2H, $C_{2'''}$-*H*); 1.42 (*t*, *J*= 7.2 Hz, 3H, $C_{2'}$-*H*); 1.24-1.18 (*m*, 22H, $C_{3'''}$-*H*-$C_{13'''}$-*H*); 0.80 (*t*, *J*= 6.9 Hz, 3H, $C_{14'''}$-*H*)

^{13}C-NMR δ (75.48 MHz, $CDCl_3$): 174.74 (*C_4*); 165.72 (*C_9*); 162.63 (*C_7*); 148.66 (*C_2*); 148.61 (*C_{8a}*); 136.89 (*C_5*); 121.53 (*C_{4a}*); 121.11 (*C_6*); 112.07 (*C_3*); 65.18 (*$C_{1'''}$*); 46.57 (*$C_{1'}$*); 31.92 (*$C_{2'''}$*); 29.69 (*$C_{3'''}$* and *$C_{4'''}$*); 29.67 (*$C_{5'''}$* and *$C_{6'''}$*); 29.63 (*$C_{7'''}$*); 29.53 (*$C_{8'''}$*); 29.36 (*$C_{9'''}$* and *$C_{10'''}$*); 28.77 (*$C_{11'''}$*); 26.00 (*$C_{12'''}$*); 25.08 (*$C_{1''}$*); 22.69 (*$C_{13'''}$*); 15.21 (*$C_{2'}$*); 14.14 (*$C_{14'''}$*)

Melting point: 69-71ºC

1-Methyloctyl 1-ethyl-1,4-dihydro-7-methyl-1,8-naphthyridine-4-oxo-3-carboxylate (10)

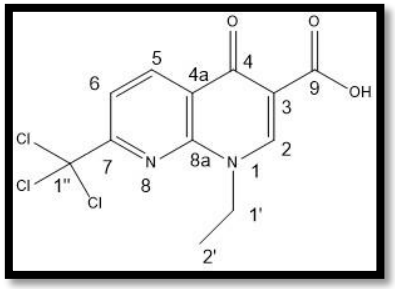


To synthesize this derivative, 16.54 mmol (1.20 mL) of thionyl chloride was added to 0.86 mmol (0.20 g) of NAL and the flask was kept aside for 15 minutes. Later 29.60 mmol (4.27 g) of 2-nonanol was added to the solution, drop by drop and mixed thoroughly after each addition, and the reaction was kept for refluxing during 1h.

Then, the excess of thionyl chloride was evaporated and the organic phase was obtained after washing with 2x10 mL of sodium chlorine, 3x10mL of sodium bicarbonate and 2x5mL of dichloromethane and drying with sodium sulfate anhydrous. Then, the solvent was evaporated and the organic phase was purified by column chromatography (7/3 to 1/1, hexane/ethyl acetate).

Yellow solid, η= 79%
^{1}H-NMR δ (300 MHz, $CDCl_3$): 8.64 (*d*, *J*= 8.1 Hz, 1H, C_5-*H*); 8.57 (*s*, 1H, C_2-*H*); 7.23 (*d*, *J*= 8.1 Hz, 1H, C_6-*H*); 5.17 (*m*, 1H, $C_{1'''}$-*H*); 4.48 (*q*, *J*= 7.2 Hz, 2H, $C_{1'}$-*H*); 2.65 (*s*, 3H, $C_{1''}$-*H*); 1.83-1.73 (m, 1H, $C_{2'''}$-*H*); 1.67-1.56 (*m*, 1H, $C_{2'''}$-*H*); 1.49 (*t*, *J*= 7.2 Hz, 3H, $C_{2'}$-*H*); 1.36 (*d*, *J*= 6.3 Hz, 3H, C*H*$_3$-$C_{1'''}$-*H*); 1.35-1.22 (*m*, 10H, $C_{3'''}$-*H*-$C_{7'''}$-*H*); 0.87 (*t*, *J*= 6.9 Hz, 3H, $C_{8'''}$-*H*)
^{13}C-NMR δ (75.48 MHz, $CDCl_3$): 174.76 (C_4); 165.07 (C_9); 162.54 (C_7); 148.62 (C_2); 148.37 (C_{8a}); 136.87 (C_5); 121.52 (C_{4a}); 121.02 (C_6); 112.49 (C_3); 71.72 ($C_{1'''}$); 46.52 ($C_{1'}$); 36.06 (CH_3-$C_{1'''}$); 31.83 ($C_{2'''}$); 29.47 ($C_{3'''}$); 29.21 ($C_{4'''}$); 25.54 ($C_{5'''}$); 25.06 ($C_{1''}$); 22.64 ($C_{6'''}$); 20.13 ($C_{7'''}$); 15.19 ($C_{2'}$); 14.09 ($C_{8'''}$)
Melting point: 75-77ºC

7-Trichloromethyl-1-ethyl-1,4-dihydro-1,8-naphtyridine-4-oxo-3-carboxylic acid (1a)



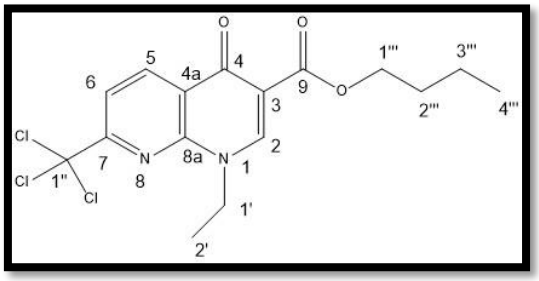
To synthesize this NAL derivative, 41.35 mmol (3.00 mL) of thionyl chloride were added to 2.15 mmol (0.5 g) of NAL. The reaction was kept under reflux for two hours, in order to form the acyl chloride. After that, the excess of thionyl chloride was evaporated.

To form the carboxylic acid, water was added to the acyl chloride and this reaction was kept under reflux overnight.

Then, the organic phase was obtained after washing 2x5 mL of dichloromethane and drying with sodium sulfate anhydrous. Then, the solvent was evaporated.

Brown solid, η= 100%
^{1}H-NMR δ (300 MHz, $CDCl_3$): 14.21 (s_{br}, C_9- O*H*); 9.03 (*s*, 1H, C_2-*H*), 8.99 (*d*, *J*= 8.4 Hz, 1H, C_5-*H*); 8.22 (*d*, *J*= 8.4 Hz, 1H, C_6-*H*); 4.67 (*q*, *J*= 7.2 Hz, 2H, $C_{1'}$-*H*); 1.63 (*t*, *J*= 7.2 Hz, 3H, $C_{2'}$-*H*)
^{13}C-NMR δ (75.48 MHz, $CDCl_3$): 178.04 (C_4); 165.85 (C_9); 161.52 (C_7); 155.21 (C_2); 149.73 (C_{8a}); 147.44 (C_5); 139.16 (C_{4a}); 121.89 (C_6); 117.84 ($C_{1''}$); 110.82 (C_3); 48.56 ($C_{1'}$); 15.09 ($C_{2'}$)
Melting point: 180-183ºC

Butyl 7-Trichloromethyl-1-ethyl-1,4-dihydro-1,8-naphthyridine-4-oxo-3-carboxylate (3a)



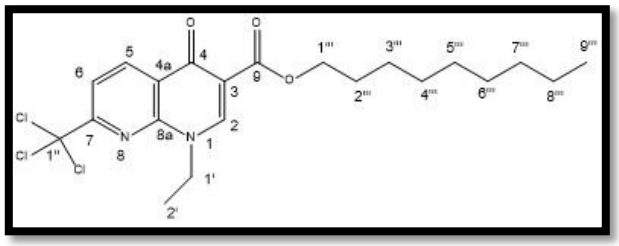
To synthetize this ester from the NAL derivative, 82.70 mmol (6.00 mL) of thionyl chloride were added to 4.31 mmol (1.00 g) of NAL. The reaction was kept in reflux for two hours, in order to form the acyl chloride. After that, the excess of thionyl chloride was evaporated.

To form the alkoxide, 4.32 mmol (0.32 g) 1-butanol were added to 32.08 mmol (0.77 g) of sodium hydride. The alkoxide was mixed with the acyl chloride and the reaction was kept under reflux for 18 hours.

Then, the organic phase was obtained after washing with 2x10 mL of sodium chlorine, 3x10mL of sodium bicarbonate and 2x5mL of ethyl acetate and drying with sodium sulfate anhydrous. Then, the solvent was evaporated and the organic phase was purified by column chromatography (9/1 to 1/1, hexane/ethyl acetate).

White solid, η= 21%
^{1}H-NMR δ (300 MHz, $CDCl_3$): 8.92 (*d*, *J*= 8.1 Hz, 1H, C_5-*H*); 8.70 (*s*, 1H, C_2-*H*); 8.06 (*d*, *J*= 8.1 Hz, 1H, C_6-*H*); 4.52 (*q*, *J*= 7.2 Hz, 2H, $C_{1'}$-*H*); 4.36 (*t*, *J*= 6.6 Hz, 2H, $C_{1'''}$-*H*); 1.79 (*m*, 2H, $C_{2'''}$-*H*); 1.57 (*t*, *J*= 7.2 Hz, 3H, $C_{2'}$-*H*); 1.48 (*m*, 2H, $C_{3'''}$-*H*); 0.99 (*t*, *J*= 7.5 Hz, 3H, $C_{4'''}$-*H*)
^{13}C-NMR δ (75.48 MHz, $CDCl_3$): 173.69 (C_4); 165.18 (C_9); 160.02 (C_7); 149.78 (C_2); 147.48 (C_{8a}); 139.55 (C_5); 124.08 (C_{4a}); 116.59 (C_6); 113.07 (C_3); 96.39 ($C_{1''}$); 65.14 ($C_{1'''}$); 47.64 ($C_{1'}$); 30.79 ($C_{2'''}$); 19.28 ($C_{3'''}$); 14.98 ($C_{2'}$); 13.83 ($C_{4'''}$)
Melting point: 113-115ºC

Nonyl 7-trichloromethyl-1-ethyl-1,4-dihydro-1,8-naphthyridine-4-oxo-3-carboxylate (5a)



To synthesize this ester, 82.70 mmol (6.00 mL) of thionyl chloride were first added to 4.35 mmol (1.01 g) of NAL. The reaction was kept under reflux for two hours, in order to form the acyl chloride. After that, the excess of thionyl chloride was evaporated.

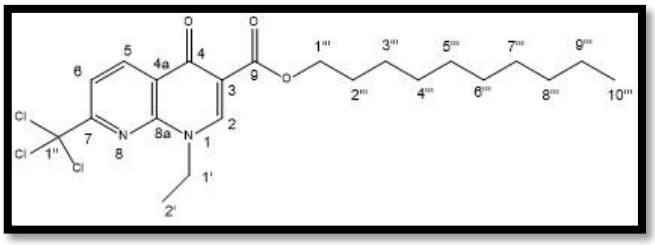
To form the alkoxide, 4.30 mmol (0.62 g) 1-nonanol were added to 6.67 mmol (0.16 g) of sodium hydride. The alkoxide was mixed with the acyl chloride and the reaction was kept under reflux for 19 hours.

Then, the organic phase was obtained after washing with 10 mL of sodium chlorine, 2x10mL of sodium bicarbonate and 5mL of dichloromethane and drying with sodium sulfate anhydrous. Then, the solvent was evaporated and the organic phase was purified by column chromatography (9/1 to 1/1, hexane/ethyl acetate).

White solid, η= 5%

^{1}H-NMR δ (300 MHz, $CDCl_3$): 8.93 (*d*, *J*= 8.1 Hz, 1H, C_5-*H*); 8.71 (*s*, 1H, C_2-*H*); 8.06 (*d*, *J*= 8.1 Hz, 1H, C_6-*H*); 4.53 (*q*, *J*= 7.2 Hz, 2H, $C_{1'}$-*H*); 4.36 (*t*, *J*= 6.9 Hz, 2H, $C_{1'''}$-*H*); 1.81 (*m*, 2H, $C_{2'''}$-*H*); 1.58 (*t*, *J*= 7.2 Hz, 3H, $C_{2'}$-*H*); 1.28 (*m*, 12H, $C_{3'''}$-*H*-$C_{8'''}$-*H*); 0.89 (*t*, *J*= 6.9 Hz, 3H, $C_{9'''}$-*H*)
^{13}C-NMR δ (75.48 MHz, $CDCl_3$): 171.71 (C_4); 165.15 (C_9); 160.03 (C_7); 149.74 (C_2); 147.48 (C_{8a}); 139.57 (C_5); 124.09 (C_{4a}); 116.58 (C_6); 113.09 (C_3); 96.40 ($C_{1''}$); 65.46 ($C_{1'''}$); 47.62 ($C_{1'}$); 31.88 ($C_{2'''}$); 29.49 ($C_{3'''}$); 29.33 ($C_{4'''}$); 29.28 ($C_{5'''}$); 28.73 ($C_{6'''}$); 25.98 ($C_{7'''}$); 22.68 ($C_{8'''}$); 14.96 ($C_{2'}$); 14.12 ($C_{9'''}$)
Melting point: 74-76ºC

Decyl 7-trichloromethyl-1-ethyl-1,4-dihydro-1,8-naphthyridine-4-oxo-3-carboxylate (6a)



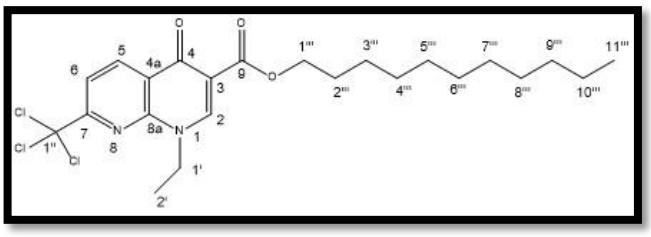
To synthesize this derivative, 0.13 mol (10.00 mL) of thionyl chloride were added to 6.02 mmol (1.39 g) of NAL. The reaction was kept under reflux for two hours, in order to form the acyl chloride. After that, the excess of thionyl chloride was evaporated.

To form the alkoxide, 4.98 mmol (0.95 mL) 1-decanol were added to 7.51 mmol (0.18 g) of sodium hydride. The alkoxide was mixed with the acyl chloride and the reaction was kept under reflux for 11 hours.

Then, the organic phase was obtained after washing with 10 mL of sodium chlorine, 2x10 mL of sodium bicarbonate and 2x5 mL of dichloromethane and drying with sodium sulfate anhydrous. Then, the solvent was evaporated and the organic phase was purified by column chromatography (9/1 to 1/1, hexane/ethyl acetate).

^{1}H-NMR δ (300 MHz, $CDCl_3$): 8.93 (*d*, *J*= 8.1 Hz, 1H, C_5-*H*); 8.71 (*s*, 1H, C_2-*H*); 8.07 (*d*, *J*= 8.1 Hz, 1H, C_6-*H*); 4.53 (*q*, *J*= 7.2 Hz, 2H, $C_{1'}$-*H*); 4.36 (*t*, *J*= 6.9 Hz, 2H, $C_{1'''}$-*H*); 1.83 (*m*, 2H, $C_{2'''}$-*H*); 1.58 (*t*, *J*= 7.2 Hz, 3H, $C_{2'}$-*H*); 1.28 (*m*, 14H, $C_{3'''}$-*H*-$C_{9'''}$-*H*); 0.89 (*t*, *J*= 6.9 Hz, 3H, $C_{10'''}$-*H*)
^{13}C-NMR δ (75.48 MHz, $CDCl_3$): 173.74 (C_4); 165.19 (C_9); 160.01 (C_7); 149.77 (C_2); 147.52 (C_{8a}); 139.59 (C_5); 124.10 (C_{4a}); 116.57 (C_6); 113.09 (C_3); 96.44 ($C_{1''}$); 65.36 ($C_{1'''}$); 47.63 ($C_{1'}$); 31.90 ($C_{2'''}$); 29.63 ($C_{3'''}$); 29.61($C_{4'''}$); 29.38 ($C_{5'''}$); 29.34 ($C_{6'''}$); 28.64 ($C_{7'''}$); 26.03($C_{8'''}$); 22.69 ($C_{9'''}$); 14.97 ($C_{2'}$); 14.12 ($C_{10'''}$)
Melting point: 69-73ºC

Undecyl 7-trichloromethyl-1-ethyl-1,4-dihydro-1,8-naphthyridine-4-oxo-3-carboxylate (7a)



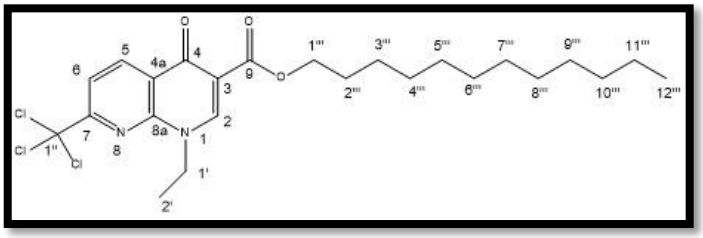
To synthesize this derivative, 82.70 mmol (6.00 mL) of thionyl chloride were added to 4.31 mmol (1.00 g) of NAL. The reaction was kept under reflux for two hours, in order to form the acyl chloride. After that, the excess of thionyl chloride was evaporated.

To form the alkoxide, 4.35 mmol (0.75 g) 1-undecanol were added to 7.08 mmol (0.17 g) of sodium hydride. The alkoxide was mixed with the acyl chloride and the reaction was kept under reflux for 11 hours.

Then, the organic phase was obtained after washing with 10 mL of sodium chlorine, 10mL of sodium bicarbonate and 2x5mL of dichloromethane and drying with sodium sulfate anhydrous. Then, the solvent was evaporated and the organic phase was purified by column chromatography (9/1 to 1/1, hexane/ethyl acetate).

Yellow solid, η= 15%
^{1}H-NMR δ (300 MHz, $CDCl_3$): 8.92 (*d*, *J*= 8.4 Hz, 1H, C_5-*H*); 8.69 (*s*, 1H, C_2-*H*); 8.06 (*d*, *J*= 8.4 Hz, 1H, C_6-*H*); 4.52 (*q*, *J*= 7.2 Hz, 2H, $C_{1'}$-*H*); 4.35 (*t*, *J*= 6.9 Hz, 2H, $C_{1'''}$-*H*); 1.80 (*m*, 2H, $C_{2'''}$-*H*); 1.57 (*t*, *J*= 7.2 Hz, 3H, $C_{2'}$-*H*); 1.26 (*m*, 16H, $C_{3'''}$-*H*-$C_{10'''}$-*H*); 0.88 (*t*, *J*= 6.9 Hz, 3H, $C_{11'''}$-*H*)
^{13}C-NMR δ (75.48 MHz, $CDCl_3$): 173.72 (C_4); 165.73 (C_9); 160.03 (C_7); 149.68 (C_2); 147.49 (C_{8a}); 139.56 (C_5); 124.09 (C_{4a}); 116.55 (C_6); 113.06 (C_3); 96.42 ($C_{1''}$); 65.43 ($C_{1'''}$); 47.65 ($C_{1'}$); 31.94 ($C_{2'''}$); 29.64 ($C_{3'''}$ and $C_{4'''}$); 29.61 ($C_{5'''}$); 29.39 ($C_{6'''}$ and $C_{7'''}$); 28.98 ($C_{8'''}$); 26.02 ($C_{9'''}$); 22.66 ($C_{10'''}$); 14.96 ($C_{2'}$); 14.13 ($C_{11'''}$)
Melting point: 77-80ºC

Dodecyl 7-trichloromethyl-1-ethyl-1,4-dihydro-1,8-naphthyridine-4-oxo-3-carboxylate (8a)



To synthesize this derivative, 0.13 mol (10.00 mL) of thionyl chloride were added to 6.03 mmol (1.40 g) of NAL. The reaction was kept under reflux for two hours, in order to form the acyl chloride. After that, the excess of thionyl chloride was evaporated.

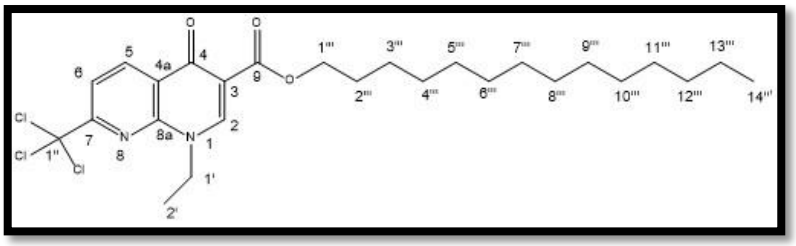
To form the alkoxide, 5.13 mmol (0.96 g) 1-dodecanol were added to 7.92 mmol (0.19 g) of sodium hydride. The alkoxide was mixed with the acyl chloride and the reaction was kept in reflux for 40 hours.

Then, the organic phase was obtained after washing with 10 mL of sodium chlorine, 10mL of sodium bicarbonate and 2x5mL of dichloromethane and drying with sodium sulfate anhydrous. Then, the solvent was evaporated and the organic phase was purified by column chromatography (9/1 to 1/1, hexane/ethyl acetate).

Yellow solid, η= 15%

^{1}H-NMR δ (300 MHz, $CDCl_3$): 8.93 (*d*, *J*= 8.4 Hz, 1H, C_5-*H*); 8.71 (*s*, 1H, C_2-*H*); 8.07 (*d*, *J*= 8.4 Hz, 1H, C_6-*H*); 4.53 (*q*, *J*= 7.2 Hz, 2H, $C_{1'}$-*H*); 4.36 (*t*, *J*= 6.9 Hz, 2H, $C_{1'''}$-*H*); 1.81 (*m*, 2H, $C_{2'''}$-*H*); 1.58 (*t*, *J*= 7.2 Hz, 3H, $C_{2'}$-*H*); 1.27 (*m*, 18H, $C_{3'''}$-*H*-$C_{11'''}$-*H*); 0.89 (*t*, *J*= 6.9 Hz, 3H, $C_{12'''}$-*H*)
^{13}C-NMR δ (75.48 MHz, $CDCl_3$): 173.86 (C_4); 165.77 (C_9); 159.99 (C_7); 149.59 (C_2); 147.65 (C_{8a}); 139.89 (C_5); 124.11 (C_{4a}); 116.58 (C_6); 113.12 (C_3); 96.35 ($C_{1''}$); 65.56 ($C_{1'''}$); 47.63 ($C_{1'}$); 31.94 ($C_{2'''}$); 29.68 ($C_{3'''}$); 29.65 ($C_{4'''}$ and $C_{5'''}$); 29.63 ($C_{6'''}$); 29.35 ($C_{7'''}$ and $C_{8'''}$); 28.87 ($C_{9'''}$); 26.02 ($C_{10'''}$); 22.71 ($C_{11'''}$); 14.94 ($C_{2'}$); 14.13 ($C_{12'''}$)
Melting point: 72-74ºC

Tetradecyl 7-trichloromethyl-1-ethyl-1,4-dihydro-1,8-naphthyridine-4-oxo-3-carboxylate (9a)



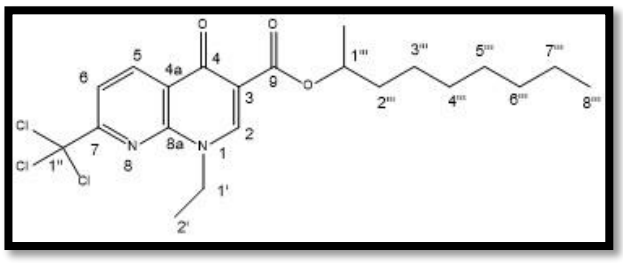
To synthesize this derivative, 82.70 mmol (6.00 mL) of thionyl chloride were added to 4.31 mmol (1.00 g) of NAL. The reaction was kept under reflux for two hours, in order to form the acyl chloride. After that, the excess of thionyl chloride was evaporated.

To form the alkoxide, 4.29 mmol (0.92 g) 1-tetradecanol were added to 30.83 mmol (0.74 g) of sodium hydride. The alkoxide was mixed with the acyl chloride and the reaction was kept under reflux for 16 hours.

Then, the organic phase was obtained after washing with 2x10 mL of sodium chlorine, 3x10mL of sodium bicarbonate and 2x5mL of ethyl acetate and drying with sodium sulfate anhydrous. Then, the solvent was evaporated and the organic phase was purified by column chromatography (9/1 to 1/1, hexane/ethyl acetate).

Yellow solid, η= 35%
^{1}H-NMR δ (300 MHz, $CDCl_3$): 8.84 (*d*, *J*= 8.1 Hz, 1H, C_5-*H*); 8.62 (*s*, 1H, C_2-*H*); 7.98 (*d*, *J*= 8.1 Hz, 1H, C_6-*H*); 4.44 (*q*, *J*= 7.2 Hz, 2H, $C_{1'}$-*H*); 4.27 (*t*, *J*= 6.9 Hz, 2H, $C_{1'''}$-*H*); 1.73 (*m*, 2H, $C_{2'''}$-*H*); 1.49 (*t*, *J*= 7.2 Hz, 3H, $C_{2'}$-*H*); 1.18 (*m*, 22H, $C_{3'''}$-*H*-$C_{13'''}$-*H*); 0.81 (*t*, *J*= 6.9 Hz, 3H, $C_{14'''}$-*H*)
^{13}C-NMR δ (75.48 MHz, $CDCl_3$): 173.69 (C_4); 165.15 (C_9); 160.03 (C_7); 149.75 (C_2); 147.49 (C_{8a}); 139.57 (C_5); 124.10 (C_{4a}); 116.57 (C_6); 113.10 (C_3); 96.41 ($C_{1''}$); 65.46 ($C_{1'''}$); 47.61 ($C_{1'}$); 31.93 ($C_{2'''}$); 29.69 ($C_{3'''}$ and $C_{4'''}$); 29.67 ($C_{5'''}$ and $C_{6'''}$); 29.63 ($C_{7'''}$); 29.54 ($C_{8'''}$); 29.37 ($C_{9'''}$); 29.33 ($C_{10'''}$); 28.73 ($C_{11'''}$); 25.98 ($C_{12'''}$); 22.70 ($C_{13'''}$); 14.96 ($C_{2'}$); 14.14 ($C_{14'''}$)
Melting point: 73-75ºC

1-Methy,octyl 7-trichloromethyl-1-ethyl-1,4-dihydro-1,8-naphthyridine-4-oxo-3-carboxylate (10a)



To synthesize this derivative, 82.70 mmol (6.00 mL) of thionyl chloride were added to 4.31 mmol (1.00 g) of NAL. The reaction was kept under reflux for two hours, in order to form the acyl chloride. After that, the excess of thionyl chloride was evaporated.

To form the alkoxide, 4.34 mmol (0.62 g) 2-nonanol were added to 30.83 mmol (0.74 g) of sodium hydride. The alkoxide was mixed with the acyl chloride and the reaction was kept under reflux for 11 hours.

Then, the organic phase was obtained after washing with 2x10 mL of sodium chlorine, 3x10mL of sodium bicarbonate and 2x5mL of ethyl acetate and drying with sodium sulfate anhydrous. Then, the solvent was evaporated and the organic phase was purified by column chromatography (9/1 to 1/1, hexane/ethyl acetate).

Yellow oil, η= 20%
^{1}H-NMR δ (300 MHz, $CDCl_3$): 8.91 (*d*, *J*= 8.4 Hz, 1H, C_5-*H*); 8.66 (*s*, 1H, C_2-*H*); 8.05 (*d*, *J*= 8.4 Hz, 1H, C_6-H); 5.18 (*m*, 1H, $C_{1'''}$-*H*); 4.52 (*q*, *J*= 7.2 Hz, 2H, $C_{1'}$-*H*); 1.79 (*m*, 1H, $C_{2''}$-H); 1.64 (*m*, 1H, $C_{2''}$-H); 1.58 (*t*, *J*= 7.2 Hz, 3H, $C_{2'}$-*H*); 1.37 (*d*, *J*= 6.3 Hz, 3H, C*H*$_3$-$C_{1'''}$-H); 1.48-1.22 (*m*, 10H, $C_{3'''}$-*H*-$C_{7'''}$-*H*); 0.87 (*t*, *J*= 6.9 Hz, 3H, $C_{8'''}$-*H*)
^{13}C-NMR δ (75.48 MHz, $CDCl_3$): 173.64 (C_4); 165.16 (C_9); 160.02 (C_7); 149.72 (C_2); 147.51 (C_{8a}); 139.60 (C_5); 124.14 (C_{4a}); 116.57 (C_6); 113.11 (C_3); 96.39 ($C_{1''}$); 71.74 ($C_{1'''}$); 47.52 ($C_{1'}$); 36.83 (*C*H$_3$-$C_{1'''}$); 31.84 ($C_{2'''}$); 29.58 ($C_{3'''}$); 29.34 ($C_{4'''}$); 25.97 ($C_{5'''}$); 22.88 ($C_{6'''}$); 20.21 ($C_{7'''}$); 14.93 ($C_{2'}$); 14.09 ($C_{8'''}$)

7.4 Ester stability assays

Kinetic studies were develop and analysed through HPLC to observe the decrease of ester concentration and the increase of acid at different times.

Before starting the kinetic studies, the right eluent for each derivative was chosen to avoid that ester's and acid's retention times were similar in order to obtain a good separation in the chromatogram. Two different eluents were chosen. For the esters 2, 3, 4 and 3a the mobile phase contained 50% of acetonitrile and 50% of water. For the other esters, the mobile phase was constituted by 85% of acetonitrile and 15% of water. All the analysis were performed with 1 mL/min flow rate, 20 µL of injection volume and a wavelength of 265 nm.

The values of k_{obs} were calculated through linear regression:

$$-k\text{obs} = \frac{\ln(conc.)t}{\ln(conc.)0}$$

(conc)t is the determinated concentrations over the time and (conc)0 is the concentration at the beginning of the assays (t=0).

7.4.1 Stability assays in phosphate buffer pH 7.4

A phosphate buffer solution was prepared with a concentration of 0.05M and ion force of 0.15M (Anex I). The assays were performed with $5x10^{-5}$M of initial concentration of substrate. At different times, samples were removed and were analysed through HPLC.

7.4.2 Stability assays in human plasma

The stability assays were performed in a solution of 80% of human plasma and 20% of phosphate buffer pH = 7.4, concentration of 0.05M and ion force of 0.15M at 37ºC. The incubation medium were field with acetonitrile (2%) and the substrate were added from stock solutions of 10^{-1} M em acetonitrile. The hydrolysis reactions were performed with $5x10^{-4}$M of initial concentration of substrate.

At specific time, 50µL of the sample was added to 450µL of acetonitrile in order to stop the hydrolysis reaction. Then, the samples were centrifuged at 15000 rpm for 10 minutes and the supernatants were analysed through HPLC.

Bibliography

7. Bibliography

1. Stanley, S. A., Raghavan, S., Hwang, W. W. & Cox, J. S. Acute infection and macrophage subversion by Mycobacterium tuberculosis require a specialized secretion system. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 13001–13006 (2003).
2. WHO. Global Tuberculosis Report 2015. *Geneva, Switz.* **1,** (2015).
3. Ahmad, S. New approaches in the diagnosis and treatment of latent tuberculosis infection. *Respir. Res.* **11,** 169 (2010).
4. Kenneth, J. R. & Ray, C. G. *Sherris Medical Microbiology*. *McGraw-Hill* (2004).
5. Bermudez, L. E., Sangari, F. J., Petrofsky, M., Goodman, J. & Kolonoski, P. The Efficiency of the Translocation of Mycobacterium tuberculosis across a Bilayer of Epithelial and Endothelial Cells as a Model of the Alveolar Wall Is a Consequence of Transport within Mononuclear Phagocytes and Invasion of Alveolar Epithelial Cells. *Am. Soc. Microbiol.* **70,** 140–146 (2002).
6. Triggle, D. J. & Taylor, J. B. *Comprehensive Medicinal Chemistry II: Volume 7: Therapeutic Areas II: Cancer, Infectious Diseases, Inflamation & Immunology and Dermatology*. *Elsevier* **7,** (2006).
7. Hokey, D. Mycobacteria. *Mycobacteria Diseases Journal* Available at: http://www.omicsonline.org/scholarly/mycobacteria-journals-articles-ppts-list.php. (Accessed: 12th October 2016)
8. UniProt. Mycobacterium tuberculosis. Available at: http://www.uniprot.org/taxonomy/1773. (Accessed: 12th October 2016)
9. Cassidy, P. M., Hedberg, K., Saulson, A., McNelly, E. & Winthrop, K. L. Nontuberculous Mycobacterial Disease Prevalence and Risk Factors: A Changing Epidemiology. *Clin. Infect. Dis.* **49,** e124–e129 (2009).
10. Woods, G. L. & Washington, J. a. Mycobacteria other than Mycobacterium tuberculosis: review of microbiologic and clinical aspects. *Rev. Infect. Dis.* **9,** 275–294 (2011).
11. Katoch, V. M. Infections due to non-tuberculous mycobacteria (NTM). *Indian J. Med. Res.* **120,** 290–304 (2004).
12. Warren, R. M. *et al.* Differentiation of *Mycobacterium tuberculosis* complex by PCR amplification of genomic regions of difference. *Int. J. Tuberc. Lung Dis.* **10,** 818–822 (2006).
13. Chatterjee, D. The mycobacterial cell wall: structure, biosynthesis and sites of drug action. *Curr. Opin. Chem. Biol.* **1,** 579–588 (1997).
14. Flynn, J. L. Immunology of tuberculosis and implications in vaccine development. *Tuberculosis* **84,** 93–101 (2004).
15. Glickman, M. S., Cox, J. S. & Jacobs, W. R. A Novel Mycolic Acid Cyclopropane Synthetase Is Required for Cording, Persistence, and Virulence of Mycobacterium tuberculosis. *Mol. Cell* **5,** 717–727 (2000).
16. Cole, S. T. *et al.* Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature* **396,** 537–544 (1998).
17. Côtes, K. *et al.* Lipolytic enzymes in Mycobacterium tuberculosis. *Appl. Microbiol. Biotechnol.* **78,** 741–749 (2008).
18. Wayne, L. G. Dormancy of Mycobacterium tuberculosis and latency of disease. *Eur. J. Clin. Microbiol. Infect. Dis.* **13,** 908–914 (1994).
19. Forrellad, M. A. *et al.* Virulence factors of the *Mycobacterium tuberculosis* complex. *Virulence* **4,** 3–66 (2013).
20. Smith, I. *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. *Clin. Microbiol. Rev.* **16,** 463–496 (2003).
21. Zumla, A., Nahid, P. & Cole, S. T. Advances in the development of new tuberculosis drugs and treatment regimens. *Nat. Rev. Drug Discov.* **12,** 388–404 (2013).
22. Mak, A. *et al.* Influence of multidrug resistance on tuberculosis treatment outcomes with standardized regimens. *Am. J. Respir. Crit. Care Med.* **178,** 306–312 (2008).
23. Rattan, A., Kalia, A. & Ahmad, N. Multidrug-resistant Mycobacterium tuberculosis: Molecular perspectives. *Emerg. Infect. Dis.* **4,** 195–209 (1998).
24. Faustini, A., Hall, A. J. & Perucci, C. A. Risk factors for multidrug resistant tuberculosis in

Europe: a systematic review. *Thorax* **61,** 158–163 (2006).
25. Lambert, N., Abdalla, A. E., Duan, X. & Xie, J. Emerging drugs and drug targets against tuberculosis. *J. Drug Target.* **0,** 1–36 (2016).
26. Dooley, K. E. *et al.* World health organization group 5 drugs for the treatment of drug-resistant tuberculosis: Unclear efficacy or untapped potential? *J. Infect. Dis.* **207,** 1352–1358 (2013).
27. DrugBank. Imipenem. Available at: https://www.drugbank.ca/drugs/DB01598. (Accessed: 23rd November 2016)
28. DrugBank. Cilastatin. Available at: https://www.drugbank.ca/drugs/DB01597. (Accessed: 23rd November 2016)
29. Ginsburg, A. S., Grosset, J. H. & Bishai, W. R. Fluoroquinolones, tuberculosis, and resistance. *Lancet Infect. Dis.* **3,** 432–442 (2003).
30. Zhang, Y., Post-Martens, K. & Denkin, S. New drug candidates and therapeutic targets for tuberculosis therapy. *Drug Discov. Today* **11,** 21–27 (2006).
31. Lienhardt, C., Vernon, A. & Raviglione, M. C. New drugs and new regimens for the treatment of tuberculosis: review of the drug development pipeline and implications for national programmes. *Curr. Opin. Pulm. Med.* **16,** 186–193 (2010).
32. Fischbach, M. A. & Walsh, C. T. Antibiotics for Emerging Pathogens. *Science (80-.).* **325,** 1089–1093 (2009).
33. DrugBank. Ciprofloxacin. Available at: https://www.drugbank.ca/drugs/DB00537. (Accessed: 12th December 2016)
34. Sharma, P. C., Jain, A., Jain, S., Pahwa, R. & Yar, M. S. Ciprofloxacin: review on developments in synthetic, analytical, and medicinal aspects. *J. Enzyme Inhib. Med. Chem.* **25,** 577–589 (2010).
35. Goldstein, E. J. C. Possible role for the new fluoroquinolones (levofloxacin, grepafloxacin, trovafloxacin, clinafloxacin, sparfloxacin, and DU-6859a) in the treatment of anaerobic infections: review of current information on efficacy and safety. *Clin. Infect. Dis.* **23 Suppl 1,** S25-30 (1996).
36. Goldstein, E. J. C. Possible role for the new fluoroquinolones (levofloxacin, grepafloxacin, trovafloxacin, clinafloxacin, sparfloxacin, and DU-6859a) in the treatment of anaerobic infections: review of current information on efficacy and safety. *Clin. Infect. Dis.* **23 Suppl 1,** S25-30 (1996).
37. DrugBank. Levofloxacin. Available at: https://www.drugbank.ca/drugs/DB01137. (Accessed: 27th December 2016)
38. DrugBank. Moxifloxacin. Available at: https://www.drugbank.ca/drugs/DB00218. (Accessed: 27th December 2016)
39. Gillespie, S. H. The role of moxifloxacin in tuberculosis therapy. *Eur. Respir. Rev.* **25,** 19–28 (2016).
40. Shen, L. L. & Pernet, A. G. Mechanism of inhibition of DNA gyrase by analogues of nalidixic acid: the target of the drugs is DNA. *Proc. Natl. Acad. Sci.* **82,** 307–311 (1984).
41. Grover, G. & Kini, S. G. Synthesis and evaluation of new quinazolone derivatives of nalidixic acid as potential antibacterial and antifungal agents. *Eur. J. Med. Chem.* **41,** 256–262 (2006).
42. DrugBank. Nalidixic Acid. Available at: http://www.drugbank.ca/drugs/DB00779. (Accessed: 18th October 2016)
43. Drlica, K. & Zhao, X. DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiol. Mol. Biol. Rev.* **61,** 377–392 (1997).
44. Aubry, A., Pan, X., Fisher, M., Jarlier, V. & Cambau, E. Mycobacterium tuberculosis DNA Gyrase: Interaction with Quinolones and Correlation with Antimycobacterial Drug Activity. *Am. Soc. Microbiol.* **48,** 1281–1288 (2004).
45. Müller, C. E. Prodrug approaches for enhancing the bioavailability of drugs with low solubility. *Chem. Biodivers.* **6,** 2071–2083 (2009).
46. Han, H. K. & Amidon, G. L. Targeted prodrug design to optimize drug delivery. *AAPS PharmSci* **2,** E6 (2000).
47. Taylor, M. D. Improved passive oral drug delivery via prodrugs. *Adv. Drug Deliv. Rev.* **19,** 131–148 (1996).
48. Silva, A. T. D. A., Chung, M. C., Castro, L. F., Güido, R. V. C. & Ferreira, E. I. Advances in

prodrug design. *Mini-Reviews Med. Chem.* **5,** 893–914 (2005).
49. Zawilska, J. B., Wojcieszak, J. & Olejniczak, A. B. Prodrugs: A challenge for the drug development. *Pharmacol. Reports* **65,** 1–14 (2013).
50. Mori, G., Chiarelli, L. R., Riccardi, G. & Pasca, M. R. New prodrugs against tuberculosis. *Elsevier* **22,** 519–525 (2017).
51. Rautio, J. *et al.* Prodrugs: design and clinical applications. *Nat. Rev. Drug Discov.* **7,** 255–270 (2008).
52. Jornada, D. *et al.* The Prodrug Approach: A Successful Tool for Improving Drug Solubility. *Molecules* **21,** 42 (2016).
53. Solomons, T. W. G. & Fryhle, C. B. *Organic Chemistry*. (Wiley, 2011).
54. Testa, B. & Mayer, J. M. *Hydrolysis in Drug and Prodrug Metabolism: Chemistry, Biochemistry and Enzymology*. (Wiley-VCH, 2003).
55. Hunt, I. Chapter 20: Carboxylic Acid Derivatives. NucleophilicAcyl Substitution. *Organic Chemistry On-Line Learning Center* (2006). Available at: http://www.chem.ucalgary.ca/courses/350/Carey5th/Ch20/ch20-3-3-1.html. (Accessed: 27th October 2016)
56. Nelson, D. L. & Cox, M. M. *Lehninger principles of biochemistry*. **33,** (W. H. Freeman, 2008).
57. Nomenclature Committee of the International Union of Biochemistry and Molecular Biology. EC 3. Introduction. Available at: http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/intro.html. (Accessed: 28th October 2016)
58. Sayali, K., Sadichha, P. & Surekha, S. Microbial Esterases: An Overview. *Int. J. Curr. Microbiol. Appl. Sci.* **2,** 135–146 (2013).
59. Bornscheuer, U. T. Microbial carboxyl esterases: Classification, properties and application in biocatalysis. *FEMS Microbiol. Rev.* **26,** 73–81 (2002).
60. Casey Laizure, S., Herring, V., Hu, Z., Witbrodt, K. & Parker, R. B. The role of human carboxylesterases in drug metabolism: Have we overlooked their importance? *Pharmacotherapy* **33,** 210–222 (2013).
61. ExPASy. ENZYME entry: EC 3.1.1.1. Available at: http://enzyme.expasy.org/EC/3.1.1.1. (Accessed: 11th November 2016)
62. Liederes, B. M. & Borchardt, R. T. Enzymes Involved in the Bioconversion of Ester-Based Prodrugs. *Wiley Intersci.* **95,** 1177–1194 (2005).
63. Li, B. *et al.* Butyrylcholinesterase, paraoxonase, and albumin esterase, but not carboxylesterase, are present in human plasma. *Biochem. Pharmacol.* **70,** 1673–1684 (2005).
64. Draganov, D. I. & La Du, B. N. Pharmacogenetics of paraoxonases: A brief review. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **369,** 78–88 (2004).
65. Adresi, Y. Butyrylcholinesterase: Structure and Physiological Importance. *Turkish J. Biochem. -Turk J Biochem.* **28,** 54–61 (2003).
66. Johnson, G. & Moore, S. W. Why has butyrylcholinesterase been retained? Structural and functional diversification in a duplicated gene. *Neurochem. Int.* **61,** 783–797 (2012).
67. Harel, M. *et al.* Structure and evolution of the serum paraoxonase family of detoxifying and anti-atherosclerotic enzymes. *Nat. Struct. Mol. Biol.* **11,** 412–419 (2004).
68. Sakurai, Y. *et al.* Esterase-Like Activity of Serum Albumin: Characterization of Its Structural Chemistry Using p-Nitrophenyl Esters as Substrates. *Pharm. Res.* **21,** 285–292 (2004).
69. Emerson Jr., T. E. Unique features of albumin: a brief review. *Critical care medicine* **17,** 690–694 (1989).
70. Guo, J. *et al.* Characterization of a Novel Esterase Rv0045c from Mycobacterium tuberculosis. *PLoS One* **5,** e13143 (2010).
71. Singh, G., Singh, G., Jadeja, D. & Kaur, J. Lipid hydrolizing enzymes in virulence: Mycobacterium tuberculosis as a model system. *Crit. Rev. Microbiol.* **36,** 259–269 (2010).
72. Fojan, P., Jonson, P. H., Petersen, M. T. N. & Petersen, S. B. What distinguishes an esterase from a lipase: a novel structural approach. *Biochimie* **82,** 1033–1041 (2000).
73. Dedieu, L., Serveau-Avesque, C., Kremer, L. & Canaan, S. Mycobacterial lipolytic enzymes: A gold mine for tuberculosis research. *Biochimie* **95,** 66–73 (2013).
74. Neves Petersen, M. T., Fojan, P. & Petersen, S. B. How do lipases and esterases work: The electrostatic contribution. *J. Biotechnol.* **85,** 115–147 (2001).

75. Canaan, S. *et al.* Expression and characterization of the protein Rv1399c from Mycobacterium tuberculosis: A novel carboxyl esterase structurally related to the HSL family. *Eur. J. Biochem.* **271,** 3953–3961 (2004).
76. Zhang, M. *et al.* Expression and characterization of the carboxyl esterase Rv3487c from Mycobacterium tuberculosis. *Protein Expr. Purif.* **42,** 59–66 (2005).
77. Simões, M. F., Valente, E., Gomez, M. J. R., Anes, E. & Constantino, L. Lipophilic pyrazinoic acid amide and ester prodrugs. Stability, activation and activity against M. tuberculosis. *Eur. J. Pharm. Sci.* **37,** 257–263 (2009).
78. Zhang, Y., Zhang, H. & Sun, Z. Susceptibility of Mycobacterium tuberculosis to weak acids. *J. Antimicrob. Chemother.* **52,** 56–60 (2003).
79. Mukaiyama, T., Usui, M., Shimada, E. & Saigo, K. A Convenient Method for the Synthesis of Carboxylic Esters. *Chem. Lett.* 1045–1048 (1975). doi:10.1246/cl.1975.1045
80. Sachdeva, S., Bhatia, S., Mittal, A. & Sinha, M. Synthesis, Evaluation and in silico studies of 1,8-Naphthyridine derivatives against antimicrobial activity. *J. Appl. Pharm. Sci.* **5,** 053–059 (2015).
81. Cynamon, M. H. *et al.* Pyrazinoic acid esters with broad spectrum in vitro antimycobacterial activity. *J. Med. Chem.* **38,** 3902–7 (1995).
82. Davis, M. & Scanlon, D. The reaction of thionyl chloride with methyl-substituted heteroaromatic compounds. *Aust. J. Chem.* **30,** 433 (1977).
83. Hoffman, E. & Stroobant, V. *Mass Spectrometry: Principles and Applications*. (Wiley, 2007).
84. Zheng, H. *et al.* Genetic basis of virulence attenuation revealed by comparative genomic analysis of Mycobacterium tuberculosis strain H37Ra versus H37Rv. *PLoS One* **3,** (2008).
85. Camus, J., Pryor, M. J., Me, C. & Cole, S. T. Re-annotation of the genome sequence of Mycobacterium tuberculosis H37Rv. *Microbiology* 2967–2973 (2017).
86. Andreu, N. *et al.* Optimisation of bioluminescent reporters for use with mycobacteria. *PLoS One* **5,** (2010).
87. Zhang, T., Li, S. Y. & Nuermberger, E. L. Autoluminescent mycobacterium tuberculosis for rapid, real-time, non-invasive assessment of drug and vaccine efficacy. *PLoS One* **7,** (2012).
88. Carey, F. A. & Sundberg, R. J. *Advanced Organic Chemistry Part A: Structure and Mechanisms*. (Springer, 2007).
89. Jacobsen, N. E. *NMR Spectroscopy Explained: Simplified Theory, Applications and Examples for Organic Chemistry and Structural Biology*. (John Wiley & Sons, 2007).
90. Field, L. D., Li, H. L. & Magill, A. M. *Organic Structures from 2D NMR Spectra*. (Wiley, 2015).
91. Balci, M. *Basic 1H- and 13C-NMR Spectroscopy*. (Elsevier, 2005).
92. Bifani, P. *et al.* Molecular Characterization of Mycobacterium tuberculosis H37Rv / Ra Variants : Distinguishing the Mycobacterial Laboratory Strain †. *J. Clin. Microbiol.* **38,** 3200–3204 (2000).
93. Ruiz, J. Mechanisms of resistance to quinolones: Target alterations, decreased accumulation and DNA gyrase protection. *J. Antimicrob. Chemother.* **51,** 1109–1117 (2003).
94. Aubry, A. *et al.* Novel gyrase mutations in quinolone-resistant and -hypersusceptible clinical isolates of Mycobacterium tuberculosis: Functional analysis of mutant enzymes. *Antimicrob. Agents Chemother.* **50,** 104–112 (2006).
95. Drlica, K. *et al.* Quinolones: Action and Resistance Updated. *Curr. Top. Med. Chem.* **9,** 981–998 (2009).
96. Patrick, G. L. *An Introduction to Medicinal Chemistry*. (Oxford University Press, 2013).
97. Smith, D. A. *et al. Pharmacokinetics and Metabolism in Drug Design*. (Wiley-VCH, 2006).
98. Pliska, V. *et al. Lipophilicity in drug action and toxicology*. (John Wiley & Sons, 1996).
99. Gu, P., Constantino, L. & Zhang, Y. Enhancement of the antituberculosis activity of weak acids by inhibitors of energy metabolism but not by anaerobiosis suggests that weak acids act differently from the front-line tuberculosis drug pyrazinamide. *J. Med. Microbiol.* **57,** 1129–1134 (2008).
100. Hu, Y., Coates, A. R. M. & Mitchison, D. A. Sterilizing activities of fluoroquinolones against rifampin-tolerant populations of Mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* **47,** 653–657 (2003).

101. Perrin, D. D. & Dempsey, B. *Buffers for PH and Metal Ion Control*. (John Wiley & Sons, 1979).

Anex

Anex I - Calculation for the preparation of a phosphate buffer solution pH 7.4, concentration 0.05M and ionic force of 0.15M

To prepare the phosphate buffer solutions with pH 7.4, concentration 0.05M and ionic force of 0.15M, it was done the following calculations based on the equations[101].

pH of the solution:

$$pKa' = pKa - (2n - 1) \times corr. <=> pKa' = 7{,}2 - 3 \times 0.1235$$

$$n = 2$$

$$pKa\ (H_2PO_4^- \leftrightarrow HPO_4^{2-}) = 7.2$$

If I = 0.15M

I = 0.1M → $corr_1$ = 0.111

I = 0.2 M → $corr_2$ = 0.136

I = 0.15 M → corr = $\frac{corr_1 + corr_2}{2}$ = 0.1235

Concentration of the solution:

$$pH = pKa' + log\frac{a}{b}$$

$$a + b = 0.05M$$

a = acidic species ($H_2PO_4^-$)
b = basic species (HPO_4^{2-})

Ionic force

$$I = \frac{1}{2} \sum_i c_i \times z_i^2$$

c – concentration of ionic species in solution
z - charge of ionic species in solution

To prepare phosphate buffer solutions with pH = 7,4, concentration of 0,05M and ionic force of 0,15M, it was added to a volumetric flask:

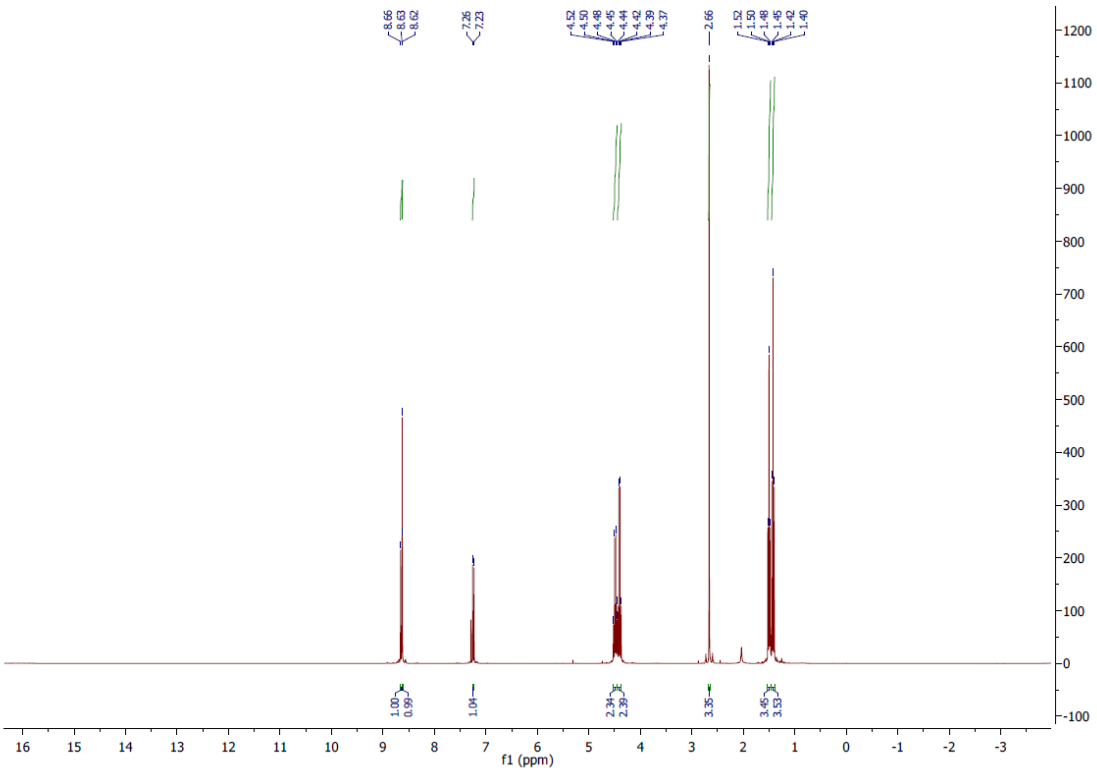
- 2.65mL of NaH_2PO_4 0.2M;
- 9.85mL of Na_2HPO_4 0.2M;
- 1.06mL of $NaClO_4$ 1M solution.

The volume obtained was 13.65 mL and the total volume of 50 mL was reached with water.

Appendix

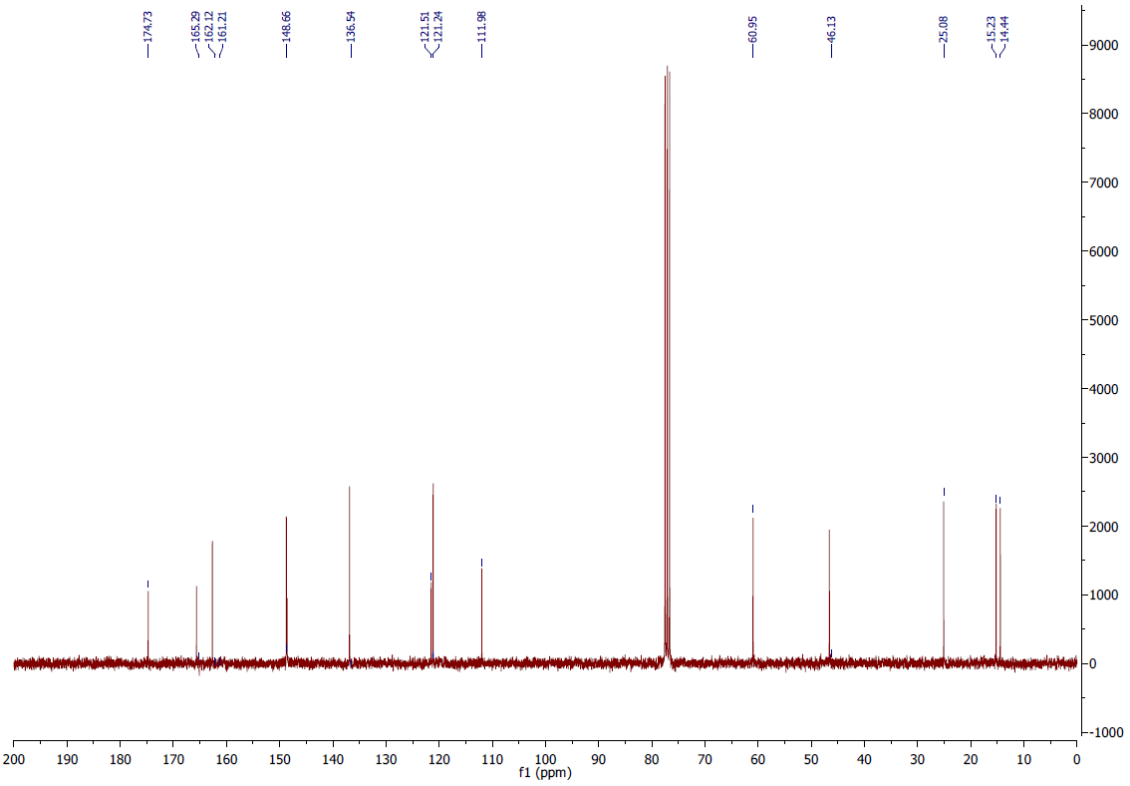
Appendix I – ^{1}H-NMR, ^{13}C-NMR, HMQC, HMBC and APT structural analysis

The followed spectras were performed in order to do the structural identification of the synthesized compounds. All the spectras were obtained in $CDCl_3$.

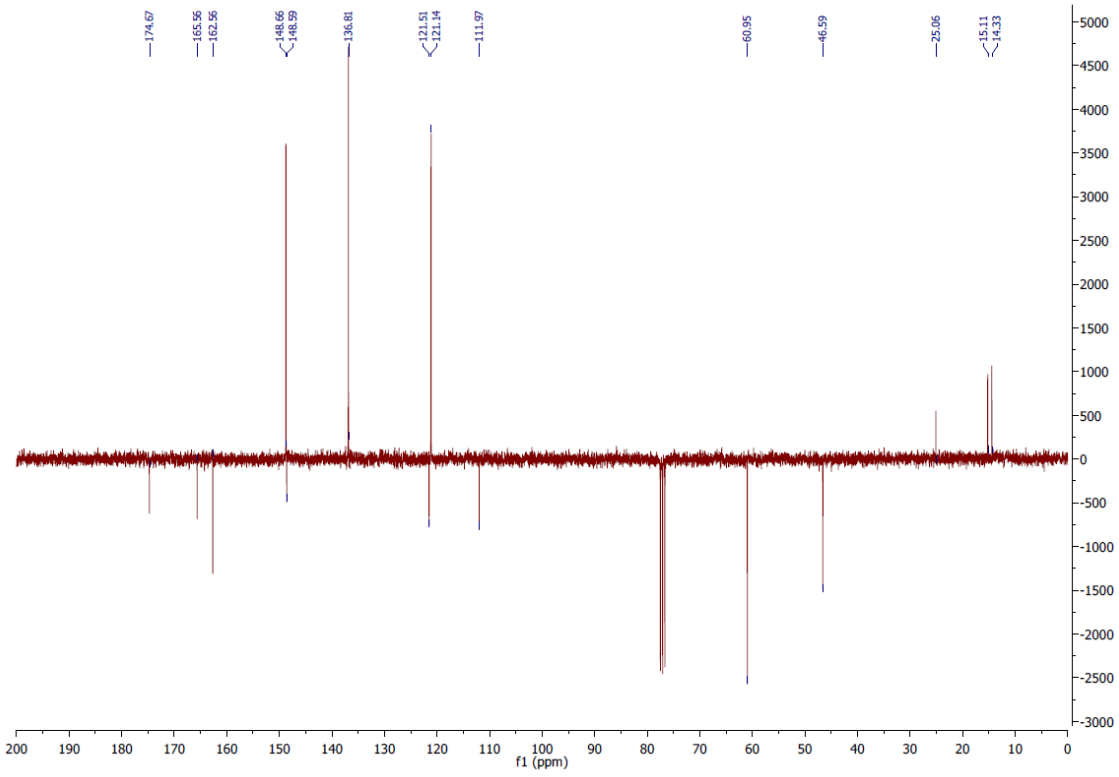


^{1}H-NMR spectra of compound 2.

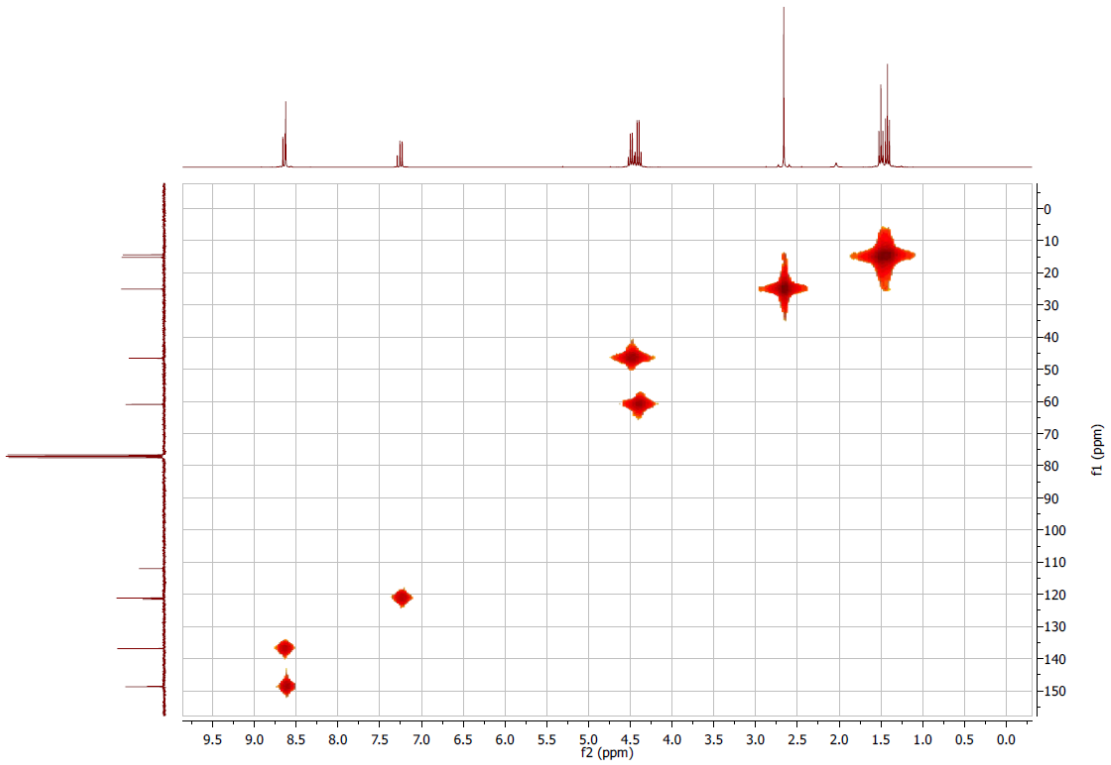
^{1}H-NMR spectra of compound 2.



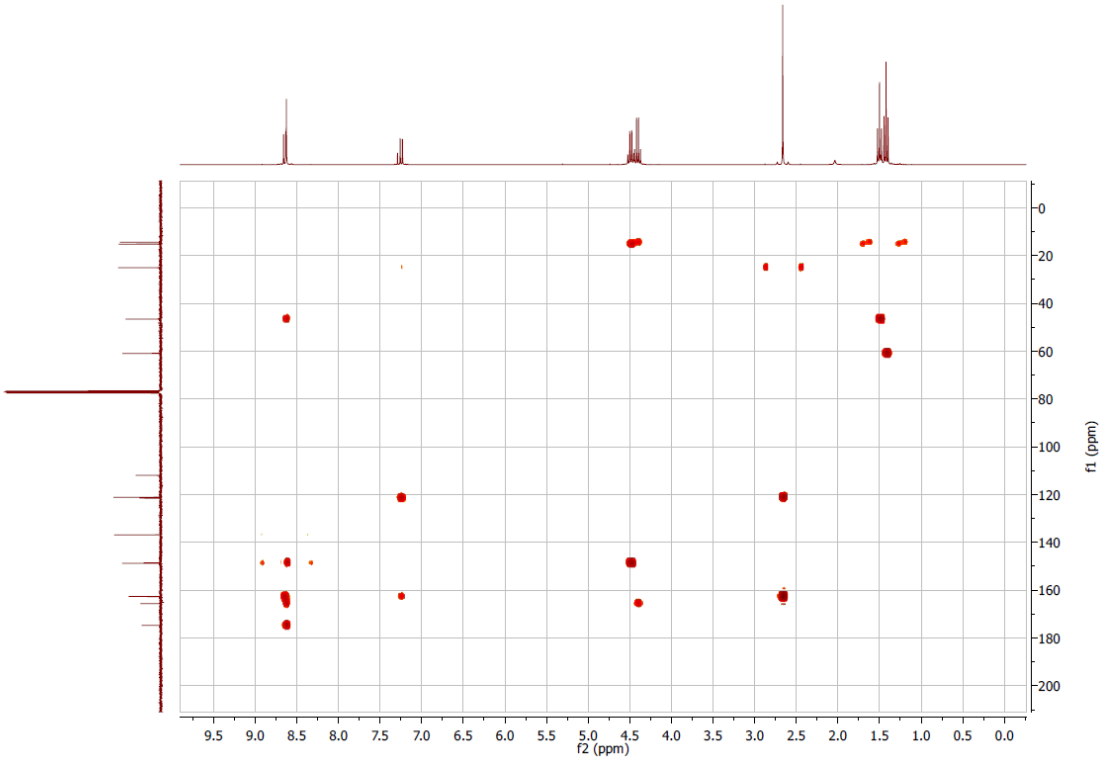
^{13}C-NMR spectra of compound 2.



APT spectra of compound 2.



HMQC spectra of compound 2.



HMBC spectra of compound 2.

Appendix II - Mobil phase used and retention time for each compound

Compounds and retention times for a mobile phase of 50:50 (ACN:Water)

Compound	R	Retention time/min
2	C_2H_5	3.7
3	C_4H_9	4.9
4	C_6H_{13}	7.7
3a	C_4H_9	8.9

Compounds and retention times for a mobile phase of 85:15 (ACN:Water)

Compound	R	Retention time/min
5	C_9H_{19}	3.8
6	$C_{10}H_{21}$	4.0
7	$C_{11}H_{23}$	4.3
8	$C_{12}H_{25}$	4.7
9	$C_{14}H_{29}$	6.4
10	$CH(CH_3)C_7H_{15}$	3.5
5a	C_9H_{19}	3.7
6a	$C_{10}H_{21}$	3.9
7a	$C_{11}H_{23}$	4.6
8a	$C_{12}H_{25}$	4.9
9a	$C_{14}H_{29}$	6.5
10a	$CH(CH_3)C_7H_{15}$	3.6